ENHANCING EFFICACY OF ONCOLYTIC MYXOMA VIRUS AND ADOPTIVE T CELL THERAPY AGAINST TUMORS

BY

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DISSERTATION

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ABSTRACT

Immunotherapy in general and adoptive cell transfer in particular have recently been in the spotlight of cancer research. Recent FDA approval of the first immunotherapeutic drugs and promising results in clinical trials suggest that this therapeutic modality holds great promise in fighting cancers. Another attractive strategy is oncolytic viral therapy, the use of cancer-specific viruses to target and kill cancer cells. Combining immunotherapies and viral therapies has shown synergistic effect in the past.

Most of our work focused on myxoma virus, a rabbit poxvirus, which can efficiently infect various types of mouse and human cancer cells. It is a strict rabbit-specific pathogen, and is thought to be safe as a therapeutic agent in all non-rabbit hosts tested including mice and humans.

Chapter 1 introduces concepts of immune system and cancer interaction, gives an overview of current immunotherapy strategies, describes the current state of oncolytic viral therapy, and ends with discussion on myxoma virus as a gene delivery agent for cancer therapy.

In chapter 2, we describe engineering a new recombinant myxoma virus (vMyx-IL15Rα-tdTr), which expresses an IL15Rα-IL15 fusion protein as well as tdTomato red fluorescent reporter protein, and the antitumor effect of the virus. Interleukin-15 (IL15) is an immunomodulatory cytokine with significant potential for stimulating anti-tumor T lymphocytes and NK cells. Co-expression of IL15 with the α subunit of IL15 receptor (IL15Rα) greatly enhances IL15 stability and bioavailability. Our findings demonstrated successful expression of the introduced proteins in cells infected by the modified virus in culture. In the animal tumor models, the novel IL15Rα-IL15 fusion protein expressing virus showed increased antitumor activity compared to non-modified virus and other controls in both $RAG1^{-/-}$ mice that lack B and T cells and in fully immunocompetent C57BL/6 mice. We showed by immunostaining that both natural killer (NK) cells and T cells likely play a role in the increased antitumor effect of vMyx-IL15Rα-tdTr.

In chapter 3, potential combination therapy of the oncolytic myxoma virus and adoptive T cell therapy was further evaluated. Combination therapy of previously characterized vMyx-IL15Rα-tdTr and naïve or tumor-antigen specific activated T cells conveyed some antitumor protection, but the effect of combination treatment was not statistically significant. Increasing the levels of tumor-associated antigens in the context of dying cancer cells sensitizes supporting cells in the tumor to reverse immunosuppression and prime antitumor adaptive immune response. We hypothesized that myxoma virus with its specificity for cancer cells could be a delivery vehicle for increasing the tumor-associated antigens at the tumor site. In order to test that hypothesis, we conducted initial steps in generating a new recombinant myxoma virus, vMyx-SIY-

tdTr, for exploring possibilities of combination therapy with the 2C T cell – SIY tumor antigen model system.

In chapter 4, we further evaluated the nature of the side effect of the adoptive T cell therapy in our preclinical model. It was noticed earlier that some tumor bearing mice treated with genetically engineered $CD4^+$ T cells developed graft-versus-host disease (GVHD) symptoms. Occurrence of these side effects is a clinically relevant phenomenon, and understanding their basis is important for improving the safety of adoptive T cell therapies. We investigated the incidence and cellular mechanism of these symptoms in experimental mice.

Overall, our work showed the feasibility of using an oncolytic/oncotropic virus as a delivery agent to additionally boost innate and adaptive immune system against cancer. Novel viral engineering strategies and therapy combinations may improve the viral as well as the immune antitumor effect. Our work has contributed to understanding the efficacy and safety of clinically relevant therapeutic approaches.

Dedicated to my Mother

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CHAPTER ONE

INTRODUCTION

1.1 Cancer and immune system

Immunotherapy is emerging as one of the most promising strategies against cancer. FDA approval of two immunotherapies, Sipuleucel-T against prostate cancer in 2010 and ipilimumab against melanoma in 2011, has driven immunotherapy to the spotlight of cancer research [1] [2]. Cancer immunotherapy was highlighted as the breakthrough of the year 2013 by the Science Magazine [3]. However, until not that long ago, skepticism about the ability of immune system to recognize cancer, let alone have the curative potential, was prevalent in scientific circles [4].

History of cancer immunotherapy

Dr. William Coley, a bone surgeon and cancer researcher from late 19th century New York, is considered to be the pioneer of cancer immunotherapy. Based on observations from historical records and previous researchers, he did clinical experiments in which he injected live *Streptococcus pyogenes* organisms in tumors of patients, some of whom experienced complete recovery. He later developed "Coley's toxin" – a vaccine adjuvant that contained a combination of two killed bacterial strains [5]. It is difficult to gauge the effectiveness of the early Coley's toxin because of less stringent standards of conducting clinical trials and scientific reporting at the time. However, it was used to treat patients with a variety of types of cancer up until the early 1950s, and some retrospective studies showed that overall survival of Coley's patients was comparable to modern conventional treatments [6] [7]. Coley's initiative to boost the immune system to fight cancer was supplanted by James Ewing and the emergence of radiotherapy starting from 1901, which remained the mainstream in cancer therapy for more than a 100 years [8]. With the incorporation of antiseptic surgical techniques in the late 1800s, establishment of radiotherapy and chemotherapy that could be more easily standardized than Coley's toxin, and increased use of antibiotics, empowerment of immune system to aid in tumor regression was considerably reduced in mainstream cancer treatment [8].

The suggestion that the immune system plays a role in tumor recognition and control gained new momentum in 1970s, with the emergence of the concept of immunosurveillance of cancer by Burnet and Thomas [9] [10]. They suggested that the immune system may protect the host from tumor development [11]. However, this hypothesis was temporarily brought into question by experiments showing that there was no difference in tumor growth rate between nude (T cell deficient) and wild type mice [12]. It was not until mouse models with more complete deficiency of the immune system were developed, that it was convincingly shown that various components of immune system have a role in tumor establishment, control and eventual disease presentation [13].

Cancer immunoediting

Robert Schreiber adapted the concept of immunosurveillance into a more general process of cancer immunoediting. This concept encompasses tumor elimination as well as sculpting the immunogenic phenotypes of tumors that eventually form in immunocompetent hosts [11]. The three E's of cancer immunoediting that describe cancer and immune system interplay are: elimination, equilibrium and escape. Elimination corresponds to immunosurveillance; equilibrium is the process in which the immune system puts selective pressure on tumor cell variants, rendering some of them capable to eventually survive immune attack; escape is the process in which tumor previously influenced by the immune system expands in an uncontrolled manner in the host [13].

Multiple components of the host immune system play roles in cancer immunoediting. In the initial steps of the elimination phase, lymphocytes of the innate (non-specific) immune response (NKT, NK and γδ T cells) recognize transformed cells and produce an immunostimulatory cytokine, IFNγ [14] [15]. Initial release of IFNγ further stimulates innate immune reactions, such as induction of chemokines that block neovascularization in the tumor, recruitment of NK cells, dendritic cells and macrophages to the tumor site, and killing of tumor cells by macrophages, NK cells and other mechanisms [16] [17] [18]. These actions result in some tumor cell death and in transport of tumor cell components to the draining lymph nodes by dendritic cells [19]. In the lymph node, the adaptive (specific) component of the immune system is raised: tumor-specific $CD4^+$ and $CD8^+$ T cells are recruited [20]. Subsequently, T cells travel to the tumor along a chemokine gradient, where they specifically recognize and destroy cells bearing antigenic peptides they have specificity for [21]. Another component of the adaptive immune system that may play a role in immunosurveillance is the humoral response, with antibodies capable of recognizing potentially mutated cancer-specific proteins expressed on the surface of the cell, and mounting an antibody-dependent cell killing response [22].

Mechanisms of tumor immunosuppression

Tolerance mechanisms are characterized by the absence of an immune response to a specific set of antigens and maintenance of normal responses to all other antigens. These mechanisms are directed against the antitumor activity elicited by cells of adaptive immune system, mainly the Th1 subpopulation of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) [23]. Examples of tolerance mechanisms induced by tumor cells include secretion of decoy receptors that bind and neutralize Fas ligand (FasL, death receptor ligand on T cells) [24], deletion of T cells by expression of death-inducing ligands on tumor cells [25], secretion of suppressive transforming growth factor-β (TGFβ) [26], inducing T cell anergy and deficient

priming etc. These processes yield to reduced or completely suppressed cytolytic activity of intratumoral effector T cells.

Additional immunosuppression mechanisms are characterized by an impaired ability of the immune system to fight cancer development, mediated by suppressive cell populations within the tumor environment, including tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), and regulatory T cells (Tregs) [23]. TAMs are a subset of macrophages that are activated towards a non-cytotoxic phenotype (M2 phenotype) [27]. They are present in the tumor environment, where they promote tumor-specific neoangiogenesis, secrete growth factors, as well as immunosuppressive cytokines like IL10 [28]. MDSCs are a heterogeneous cell population of incompletely matured granulocytes, macrophages and dendritic cells [29]. They can inactivate both $CD4^+$ and $CD8^+$ T cells, by mechanisms such as: overexpressing CD80 (B7-1), a ligand for the inhibitory molecule CTLA-4, producing the enzyme arginase, producing high levels of ROS and NO [30]. Tregs ($CD4^+CD25^+FoxP3^+$ T cells) suppress antitumor immune response through high surface expression of CTLA-4, activation of the FoxP3 transcription factor, secretion of IL10 and other mechanisms [31].

1.2 Current approaches in cancer immunotherapy

Current immunotherapy strategies include monoclonal antibodies against tumor cells or immune-regulatory molecules, cancer vaccines, cytokines and cell-based therapies such as adoptive transfer of T cells [32].

Monoclonal antibodies

Monoclonal antibodies were one of the first promising immunotherapy strategies. When hybridoma technology, invented by Kohler and Milstein in 1975, made production of monoclonal antibodies possible, hopes were high that antibodies were the "magic bullet" that would cure all cancers, as first proposed by Paul Ehrlich in 1906 [33]. Unfortunately, the clinical applicability of these first monoclonal antibodies was limited since they were made in mice; they were immunogenic in humans and had poor abilities to induce human effector responses. Advances in antibody engineering provided the possibility to make chimeric, humanized and fully human monoclonal antibodies that addressed many of these early issues [34].

In the past decade, the clinical relevance of antibodies has been evident by FDA approval of more than ten drugs that target various antigens on the tumors and tumor environment [34]. Antibodies may target surface antigens on tumor cells; for example, rituximab targets CD20 in non-Hodgkin B cell lymphoma, trastuzumab targets HER2 in breast cancer, and cetuximab targets EGFR in colorectal cancer [35] [36] [37]. These antibodies induce tumor cell death by blocking activation of growth and survival pathways, and engaging innate immune effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) [38].

Some of the recent successes in antibody-based strategies stem from targeting the tumor environment and enhancing an antitumor immune response. One goal of these approaches is reversing tumor-mediated immunosuppression by targeting immunoregulatory co-receptors [39]. FDA-approved ipilimumab antagonizes the inhibitory receptor CTLA-4, expressed on activated T cells [40]. Several antibodies blocking the PD-1 and PD-1 ligand T-cell inhibitory signaling axis are in development and on the track for FDA approval [41]. Other approaches for enhancing T cell-specific immunity against tumors aim to activate stimulatory receptors, such as 4-1BB, OX40, CD27, CD40, and DR3 [42].

Cancer vaccines

Cancer vaccines aim to induce tumor-specific T cells by introducing a tumor antigen together with an adjuvant. Ideally, therapeutic vaccines should eliminate tumors both by activating cytotoxic T cells and inducing a tumor-specific T cell memory that would prevent future tumor relapse [32]. Dendritic cells are one of the main candidates for vaccination vehicles, since their role is to bridge the innate and the adaptive immune response by stimulating T cells via antigen presentation [43]. Activated (mature) dendritic cells

are extremely efficient at antigen presentation and activating T cells compared to other antigen presenting cells and tumor cells [44]. Various approaches for exploiting dendritic cells for vaccination against cancer are explored in ongoing clinical trials [32]. One approach uses vaccines consisting of non-targeted peptide, protein or nucleic acid to be captured by patient's dendritic cells, and these agents are frequently combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) or various adjuvants such as toll-like receptor (TLR) agonists [45] [46]. A second approach is targeting the dendritic cells *in vivo* via coupling antigens to antibodies specific to surface receptors on dendritic cells [47]. Agents that are dendritic cell activators such as TLR3, TLR7-8, or CD40 agonists enable their maturation and activation [48]. Finally, a third approach is vaccination with *ex vivo* generated dendritic cells, which has been studied for many years and has recently resulted in FDA approval of the first therapeutic cancer vaccine Sipuleucel-T (PROVENGE by Dendreon) [1] [49]. Sipuleucel-T is approved for metastatic prostate cancer and it consists of enriched blood antigen presenting cells cultured with a fusion protein of prostatic acid phosphatase (PAP) and GM-CSF [50].

Cytokines

Cytokines are molecular messengers that can directly stimulate immune cells at the tumor site. They are secreted or membrane-bound proteins that are produced by cells of the innate and adaptive immune system in response to pathogens and tumor antigens [51]. Two cytokines were so far granted FDA approval as single agents for cancer treatment: IL2 for metastatic melanoma and renal cell carcinoma, and IFNα as an adjuvant therapy for stage III melanoma [52] [53]. Other cytokines have shown broad antitumor activity in preclinical models, and cytokines such as GM-CSF, IL7, IL12, IL15, IL18 and IL21 are being evaluated in clinical trials for patients with cancer [54] [55] [56] [57] [58]. Other efforts are focusing on neutralization of immunosuppressive cytokines, IL10 and TGFβ [59]. However, multiple roles of single cytokines in both immune activation and suppression, redundancy of cytokine signaling, and high potency of these agents makes it challenging to achieve high antitumor responses without causing toxicities. The full potential of cytokines likely lies in their use as components of combination therapy, together with small molecule inhibitors, vaccines, monoclonal antibodies or other agents that can reverse immunosuppression at the tumor site [60].

1.3 Adoptive cell therapy

Adoptive cell therapy (ACT) involves the isolation and reinfusion of T cells with antitumor activity into cancer patients. Transferred cells can be either endogenous antitumor T cells or they can be genetically engineered to express receptors with antitumor activity [61]. Adoptive T cell therapy has shown remarkable effectiveness in clinical trials for certain cancers; for example 20-40% patients with metastatic melanoma can be cured using this approach, whereas the complete response rate for other treatments is 1-6% [62]. Objective response rates between 51% to 72% have been reported in heavily pretreated advanced melanoma patients [62].

Tumor infiltrating lymphocytes

The first adoptive transfer cancer therapies were pioneered by Steven Rosenberg's group at the National Cancer Institute (NCI) in Maryland. They were done with patients' tumor infiltrating lymphocytes (TILs), grown from resected melanomas with the addition of IL2 [63]. These tumor-resident T cells can retain reactivity against endogenous tumor-associated antigens, and are effective in combination with a lymphodepleting preparative regimen and administration of IL2, with 22% of treated patients achieving a complete tumor regression [62]. Therapeutic success of TILs in melanoma is partially due to the high immunogenicity of these tumors, probably related to the fact that melanoma has the highest mutation rate of any cancer type [64]. Identifying T cells capable of recognizing cancer cells beyond melanoma without causing deleterious effects on normal cells is the major challenge for broad application of ACT [61].

Genetically engineered T cells

In order to empower T cells to recognize and eliminate cancers beyond melanoma, genetic engineering of patients' lymphocytes using genes for antitumor receptors is under active research. Two types of receptors are currently developed for this purpose: variants of conventional T cell receptors (TCRs) and chimeric antigen receptors (CARs) [61]. TCRs are molecules naturally found on all T cells; they are heterodimers that are able to recognize peptides originating from intracellular proteins in the context of major histocompatibility complex (MHC) molecules on the cell surface [65] [66]. On the other hand, CARs do not occur naturally; they are chimeras between the antigen-recognizing portion of an antibody attached to intracellular T cell signaling domains such as CD3ζ, CD28 and 41BB. The introduction of CARs enables T cells to recognize an antigen the same way antibodies do - without MHC restriction and with capability of recognizing a wider variety of structures [67]. Genes for TCRs and CARs with favorable antitumor activity can be inserted into retroviruses or lentiviruses which can transform lymphocytes from patients and convert them into cells capable of killing tumors [68].

Differentiation state and activity of tumor specific T cells

Emerging findings from both preclinical studies and clinical trials highlight the importance of the differentiation state of the adoptively transferred T cell populations to the success of ACT [69]. Upon their activation, T cells begin to proliferate and differentiate, and they need to be fully differentiated for optimal antitumor efficacy [70]. However, it is evident that T cell differentiation is inversely correlated with persistence and antitumor efficacy of transferred cells. For $CD8^+$ T cells, T memory stem (T_{SCM}) cells are more effective against tumors than central memory T (T_{CM}) cells, which are more effective than effector memory T (T_{EM}) cells [71]. Recently, $CD8^+$ T memory stem (T_{SCM}) cells are emerging as a favorable developmental stage for ACT, since they proliferate extensively and they can further differentiate into T_{CM} and T_{EM} cells [72].

A lymphodepleting regimen that removes immunosuppressive cellular elements within the tumor microenvironment before ACT results in higher levels of durable responses in patients [73]. Lymphodepletion is a temporary ablation of the immune system in a patient with cancer, accomplished using chemotherapy alone or in combination with total-body irradiation [69]. Several mechanisms might contribute to the enhanced efficacy of transferred T cells in the lymphodepleted host environment. These mechanisms include elimination or reprogramming of myeloid derived suppressor cells (MDSCs) [74], elimination of suppressive regulatory T cells (Tregs) [75], depletion of endogenous lymphocytes that compete for homeostatic cytokines [76], and increased functionality of APCs, partly mediated by bacterial translocation across intestinal epithelium accompanying total-body irradiation [77].

Choosing the right targets for ACT

The identification of appropriate tumor antigens is perhaps the greatest challenge now facing the field of cancer immunotherapy [78]. Potentially targetable tumor-associated antigens that T cells can recognize fall into five major categories [69]:

First, unaltered tissue-differentiation antigens shared between the tumor and the non-malignant cells that the tumor originated from can be used as a target, as long as the antigen is present on tissues non-essential for life. The biggest success of ACT with engineered T cells has been seen with targeting an antigen from this category, CD19. CD19 is expressed on B cell lymphomas, as well as on normal B cells [79]. Other antigens in this category include carcinoembryonic antigen (CEA) (present on colon cancer and normal colon) [66] and melanocyte differentiation antigens (MDAs) — such as PMEL (also known as gp100), melanoma antigen recognized by T cells 1 (MART1), tyrosinase, tyrosinase-related protein 1 (TYRP1) and TYRP2 (on most melanoma tumors but also on normal melanocytes) [80].

In the second category of targetable antigens are products of mutated genes, since T cells potentially recognizing these neo-antigens were not subject to central tolerance. High incidence of mutations is particularly characteristic for melanoma [81]. The third category includes viral antigens, for cancers associated with virus infections, such as those derived from Epstein–Barr virus and human papillomavirus (HPV) [82] [83]. The fourth category includes antigens produced by epigenetic changes, most notably cancer-testis antigens that are products of genes inactive in normal non-germline tissues. Examples include NY-ESO-1, MAGEA3 and synovial sarcoma X breakpoint 2 (SSX2) [84] [85]. Finally, the fifth category includes targetable antigens on non-transformed tumor vasculature and stroma that support tumor sustenance; examples include fibroblast activation protein (FAP) and vascular endothelial growth factor receptor 2 (VEGFR2), expressed at higher levels at tumor neo-vasculature [86] [87].

ACT clinical trials: successes and challenges

Recent clinical trials have revealed successes as well as drawbacks of targeting previously discussed tumor-associated antigens. CD19 is an almost ideal target for cancer therapy – apart from cancer cells, it is present only on B cells in the body, which are necessary for normal life but not essential for patient's survival [88]. The success of anti-CD19 CAR therapy is one of the reasons for the 2013 Science Magazine designation of breakthrough of the year for cancer immunotherapy [3]. Some of the successful recoveries were the topic of national news, like the story of the first pediatric patient with acute lymphoid leukemia (ALL) to be treated with genetically modified T cells at the University of Pennsylvania by the team of Dr. Carl June [89] [90]. Unfortunately, this type of target is very difficult to find in other types of cancers, and treating solid tumors presents with an additional share of challenges.

A recent clinical milestone from Dr. Rosenberg's group was a tumor regression in a patient with metastatic epithelial cancer treated with TILs enriched for T cells recognizing a mutation in their own cancer, identified by a whole-exomic-sequencing-based approach [91]. This is the first reported instance of T cell therapy tailored to specific mutations in patient's cancer; however, the reported results are from a single patient study, so conclusions should be cautiously extrapolated.

Clinical trials have shown undesirable, sometimes even detrimental autoimmune effects of targeting certain tumor-associated antigens initially considered favorable. T cells targeting MART1 caused toxicity to melanocytes in the skin, eye and ears in almost all treated patients, and had limited antitumor efficacy [65]. Similarly, T cells targeting carcinoembryonic antigen (CEA) resulted in partial response in one of three patients with metastatic colorectal cancer; however dose-limiting diarrhea due to autoimmune colitis developed in all treated patients [66]. Some of the adverse effects of ACT with engineered T cells resulted from unanticipated cross-reactivity of the TCRs with unintended antigens ("off tumor, off target" toxicity).

Notably, a TCR specific for an epitope from the cancer-testis antigen MAGEA3 caused severe neurological toxicities in patients, because the TCR unexpectedly recognized a nonidentical epitope of a brain protein, MAGEA12 [92]. Another trial targeting MAGEA3 with a different TCR reported fatal cardiac toxicity, shown to be due to the cross reactivity of engineered T cells with a normal myocardial protein titin [93].

A lot of these targets were previously targeted by cancer vaccines, without significant autoimmunity. This highlights the potency of the ACT approach, and also a need for careful evaluation of TCR and CAR cross-reactivity with self-antigens and determining the clinical relevance of even very low levels of target expression in normal tissues [94].

Concluding remarks on ACT

Despite cautious optimism prevalent in cancer immunotherapy circles, there is still a long way to go before ACT for cancer becomes safe and available on a larger scale. Like any personalized therapy, the logistics and cost of having to propagate and manipulate individuals' cells might be a prohibitive factor in the application of this therapeutic modality [95]. However, with recent involvement of the big pharmaceutical companies like Novartis in the development of the infrastructure required for the widespread availability of this technology and a high likelihood of TCRs and CARs being available "off the shelf", some challenges of this kind might be circumvented in the foreseeable future [68]. Efficacy of ACT may also be augmented with combination therapies, such as therapeutic vaccination, checkpoint inhibition, agonistic antibodies, small molecule inhibitors of tumors, and targeting of tumor stroma and neo-vasculature. Finding the optimal targets and the right regimen for successful T cell therapy remains the main challenge to broad anticancer application of ACT.

1.4 Oncolytic virotherapy

Oncolytic virotherapy is emerging as a promising antitumor strategy that has immunological dimensions. The safety of several virus platforms has been confirmed in early-stage clinical trials, and viruses from three families (herpes-, pox- and adenoviruses) are currently being evaluated in advanced clinical trials [96]. New genetic engineering strategies have led to improved systemic administration, better tumor specificity and improved antitumor efficacy [97].

Oncolytic viruses are therapeutically useful viruses that can selectively infect and destroy cancerous tissues without causing harm to normal tissues [98]. Cancer cells are in general more susceptible to viruses than their nontransformed cellular counterparts, since during the malignant transformation they often lose the potent innate antiviral response pathways, including type I and II interferon (IFN) and tumor necrosis factor (TNF) responses [99]. Viruses have evolved specificities for different cell types, and this natural diversity is useful for developing therapies for different tumors; hence, multiple viral families (currently nine) are being evaluated in clinical trials [100].

Specific tropism of an oncolytic virus to the cancer cell is an essential requirement for successful cancer therapy. Certain viruses exhibit an inherent preference for malignant cells, and others are engineered for improved oncotropism [99]. The first generation of engineered viruses had their virulence factors deleted to attenuate replication in normal cells; however this reduced their replication and effectiveness in cancer cells as well. The next generation of engineered viruses focused on methods of enhancing specificity of the viruses to cancer cells with improving their antitumor potency [97].

Based on the results from current clinical and preclinical trials, it is no longer regarded as essential for the therapeutic virus to directly infect and kill every cancer cell in the patient. It is becoming evident that antitumor immune responses induced by viral therapy are key determinants of therapy success [101]. Oncolytic viruses can cooperate with host immune system in a number of ways; viruses can be delivery vehicles for death signals specific for cancer cells, they can be used as gene therapy vectors for expression of anticancer genes, or they can upregulate antitumor immunity via their oncotropic immunostimulatory properties [102].

Brief history of oncolytic virotherapy

The history of using pathogens as antitumor reagents starts with history of immunotherapy and Dr. Coley's experiments, as addressed above. A number of early clinical trials conducted in 1950s and 1960s used impure oncolytic virus preparations, including body fluids containing human or animal viruses; they generally failed to impact tumor growth, and had high morbidity rate [103]. Despite occasional successes,

such as tumor regressions in 37 out of 90 terminal cancer patients treated with mumps virus at Osaka University, this work was not pursued beyond the 1970s [104]. The re-emergence of oncoviral therapeutic strategies came with the advent of genetic engineering and the ability to improve tumor-specificity and other aspects of viral antitumor properties [105]. Since a landmark study in which a thymidine kinase–negative Herpes Simplex Virus (HSV) with attenuated neurovirulence was shown to be active in a murine glioblastoma was published in 1991 [106], dozens of different viruses have been engineered for cancer therapy.

Overview of current clinical trials

The oncolytic virus that has had most notable success in clinical trials so far is a modified herpesvirus, Talimogene laherparepvec (T-VEC, formerly OncoVex, developed by Amgen). T-VEC is an oncolytic HSV genetically manipulated to drive the expression of human granulocyte macrophage–colony stimulating factor (GM-CSF) by infected cells [107]. In the phase II clinical trial, this virus was administered by direct intratumoral injection to patients with metastatic malignant melanoma and led to complete regression of lesions in 8 of 50 treated patients [108]. Tumor shrinkage was noted in injected as well as non-injected lesions, demonstrating that systemic immunity was induced [109]. It was recently announced that the phase III trial, in which T-VEC effect was compared to GM-CSF alone, has met its primary endpoint of durable response rate (complete or partial response lasting for at least six months); the T-VEC group had 10.8% complete responders in comparison to 0.7% complete response rate in the GM-CSF alone group [102]. These results suggest that T-VEC might be close to FDA approval for use in melanoma patients [96].

Another notable example of an oncolytic virus in the late stage of clinical testing is a poxvirus JX954 (developed by Transgene as Pexa-Vec), an oncolytic vaccinia virus also engineered to express GM-CSF [110]. In the completed phase II clinical trial, JX594 was shown to induce objective responses in 15% of hepatocellular carcinoma patients [111]. Eight current clinical trials are assessing the safety and antitumor profile of JX594 for hepatocellular carcinoma and other cancers [96].

In addition to these two notable examples, there have been published clinical studies using a variety of other viruses, including attenuated adenoviral strains, Newcastle disease virus for glioma and other solid tumors, parvovirus for glioblastoma, and reovirus for glioma, melanoma and other solid tumors. The majority of these studies were phase I/II trials, most often reporting favorable safety data and sporadic antitumor activity. Currently, there are 52 ongoing clinical trials assessing the safety and antitumor potential of oncolytic viruses from ten different viral families in cancer patients [96].

Improving viral antitumor potential

Despite the promising results, oncolytic viruses that have been used in advanced clinical trials have not met efficacy milestones expected from preclinical models. Based on lessons learned, current preclinical studies are focusing on resisting virus neutralization, improving tumor specificity, arming the virus for improved antitumor activity, and exploring different combination therapies [97]. Our own preclinical work has employed an oncolytic virus, myxoma virus, to deliver a cytokine gene to engender the local secretion within the tumor of a potent cytokine that activates cytotoxic T cells and natural killer cells.

Resisting neutralization

Intravenous administration of oncolytic viruses is the preferred administration route for the treatment of metastatic disease; however systemic delivery makes the virus susceptible to neutralization by pre-existing antibodies in the blood and by the mononuclear phagocytic system (MPS) of liver and spleen [96]. Genetic engineering strategies are developed for shielding the viruses by changing or physically masking the epitopes recognized by antibodies. Modifications have been made so far for vesicular stomatitis virus (VSV), adenovirus and measles virus [112] [113]. Altering the antibody recognition epitopes in oncolytic viruses as well as regimen of combination therapy with pharmacological immunosuppression should help in effectively initiating viral antitumor action [99].

Tumor selectivity

Virus selectivity for cancer cells can be enhanced either on the virus entry level (virus recognizing surface proteins) or post-entry on the virus replication level [97]. Entry targeting is improved by fusion or conjugation of specificity domains with virus entry receptors, for example retargeting HSV to breast cancer cells by engineering its glycoprotein D to express single-chain antibody for HER2 [114]. Tumor-replication targeting takes advantage of altered gene expression in cancer cells and it involves using promoters and engineered microRNA target sequences. For example, herpesviruses have been engineered to express wild-type virulence factors under hepatocellular carcinoma-specific promoters [115], and a number of viruses have been modified to express miRNA target sequences that will stop their replication in normal cells (certain miRNAs have abnormally low expression in tumor cells) [116]. These novel approaches minimize off-target toxicities while preserving potent virus replication in cancer cells [117].

Arming the virus

Ability to access all cells within a tumor remains a clinical challenge even for viruses that can specifically and effectively infect the tumor. A desirable consequence of productive virus infection would be induction of "bystander cell killing", where the virus would cause destruction of surrounding cells that are not directly

infected [97]. Different strategies have been employed to arm the viruses to induce this remote destruction. First, viruses armed with prodrug convertases can be used to activate prodrugs used for chemotherapy, as in the combination of adenovirus with the purine nucleoside phosphorylase (PNP) transgene with prodrug fludarabine [118], used in a current clinical trial. Second, viruses that express ion transport proteins can increase radiation poisoning when combined with radioisotopes, such as measles virus expressing human sodium–iodide symporter (NIS) in combination with radioactive iodine [119]. Third, viruses are engineered to express immunostimulatory factors can induce innate and adaptive immune responses to tumor-associated antigens, most famously so far with viruses expressing GM-CSF to stimulate the production of granulocytes and monocytes, which in turn stimulate adaptive immunity [120] [121].

Combination therapies

Immune checkpoint blockade strategies, such as blocking immune inhibitory molecules CTLA-4, PD-1, PD-L1, are currently being explored as combinatorial therapeutics with oncolytic viruses [122]. For example, T-VAC is being tested in combination with the anti-CTLA-4 antibody ipilimumab [96]. In addition to combinations with viruses engineered to express prodrug activating enzymes, a number of chemotherapeutic drugs in use are potentially synergistic with oncolytic viruses [123]. For example, the nucleoside analog gemcitabine increases the efficacy of certain viruses [124].

1.5 Oncolytic myxoma virus for gene delivery

Another way that an oncolytic virus can be combined with other approaches is to use the virus to selectively deliver a gene to cancer cells in order to have a protein expressed only in the tumor. For example, virally delivered genes for cytokines could promote the responses of immune cells within the tumor environment and avoid systemic toxicity from inflammatory actions of the cytokine. We have explored the use of the oncolytic myxoma virus for this purpose.

Myxoma virus is a poxvirus that infects only rabbits and European Brown Hares in the wild, and is nonpathogenic in all other animals tested [125]. It causes lethal disease called myxomatosis in the European rabbit and it was used in the 1950's in an attempt to control the Australian feral rabbit population [126]. Despite its lack of broad pathogenicity, myxoma virus can replicate in cultured cells from many species, including most human cancer cells which are particularly permissive for the virus [127] [128] [129].

Myxoma has several advantages over other oncolytic viruses: humans have no prior immune response against it; it is completely safe in humans and mice; it replicates in the cytoplasm and does not integrate into genomic DNA [130]. Its large linear double stranded DNA genome consisting of 159 unique viral genes allows for as much as 25kb of contiguous DNA to be inserted, therefore enabling the expression of potential therapeutic and reporter genes [125] [127].

Antitumor properties of myxoma virus were shown in a number of preclinical studies since the first reports of its oncolytic activity. Lun et al. first demonstrated oncolytic activity of myxoma virus *in vivo* in 2005; they showed that myxoma virus cured human glioma xenografts in 92% virus-treated nude mice [131]. Activity in human xenograft model in nude mice was also showed for rhabdoid tumors [132]. Human medulloblastoma xenograft mouse models showed prolonged survival upon treatment with myxoma virus, and animal survival was further enhanced with the combination treatment with rapamycin, an mTOR inhibitor [133]. Oncolytic potency of myxoma virus in syngeneic tumor models was shown by Stanford et al.: virus treatment caused growth inhibition of primary B16-F10 melanoma tumor, as well as reduced number of lung tumor burden in the metastatic model [134].

Binding and entry by poxviruses into mammalian cells is generally not restricted at the cell surface level, contrary to some other oncolytic viruses. The mechanism of specificity and cell entry, even for the well-characterized poxviruses such as vaccinia virus, was unknown until recently. It is now known that vaccinia induces cellular uptake via endocytosis triggered by exposed phosphatidylserine residues on the viral membrane, mimicking absorption of apoptotic cells [135]. It has been shown recently that vaccinia and

myxoma exploit different mechanisms to enter and infect human cancer cells, which provides some rationale for their divergent cancer cell tropisms [136].

Non-permissive cells can abort the replication cycle of the virus, while signaling defects in cancer cells permit productive viral replication. Tropism of myxoma virus to human cancer cells is linked to hyperactivation of serine/threonine kinase Akt in these cells [129]. A viral host range protein M-T5 has been shown to regulate the ability of myxoma virus to propagate in human tumor cells [137]. M-T5 forms complexes with two human intracellular proteins: Cullin-1 and Akt-1. The interaction of M-T5 with Cullin-1, an E3 ubiquitin ligase involved in cell cycling, prevents cell cycle arrest and apoptosis to allow viral replication. Binding to M-T5 activates Akt, an important serine/threonine kinase involved in survival, proliferation and cell death. Permissiveness of myxoma infection is directly dependent on level of phosphorylation (activation) of Akt. Many human cancers exhibit hyperactivated Akt. The molecular mechanism of Akt/MT-5 interaction is under active investigation [130].

Rapamycin, an immunosuppressant with antitumor effects, has the ability to increase the oncolytic potential of myxoma virus [133]. Rapamycin acts directly on the mammalian target of rapamycin (mTOR1) and has the ability to increase viral replication that correlates with Akt activation. [138] [139]. Treatment of semi-permissible, but not fully permissible or non-permissible, human cancer cells with rapamycin increases viral replication and spread in vitro [138]. These results suggest that pre-treatment with this drug may be necessary for productive tumor infection by myxoma virus *in vivo* [134] [140]. Furthermore, results from our laboratory show that rapamycin treatment did not impair T cell-mediated tumor destruction, supporting the feasibility of combining adoptive immunotherapy and rapamycin-enhanced virotherapy [141].

Interleukin 15 (IL15) and IL15Rα

Discovery and characterization of cytokine IL2 provided an important stimulus to the development of cancer immunotherapy [61]. Clinical trials conducted from the 1980's to present day showed the ability of high-dose IL2 to mediate durable complete regressions, and it was approved by the FDA for the treatment of patients with metastatic renal cancer in 1992 and metastatic melanoma in 1998 [61]. Today, other recombinant cytokines and hematopoietic growth factors are also approved for use as supportive agents in cancer, notably interferon-α (IFNα) for the surgical adjuvant treatment of high-risk malignant melanoma [142].

Interleukin 15 (IL15) was at the top of the National Cancer Institute's list of agents with the greatest potential use in tumor immunotherapy in 2007 [143], and currently five cancer clinical trials using IL15 are ongoing or recently completed [144]. Notably, one of the clinical trials uses recombinant human IL15 as a monotherapy for refractory metastatic melanoma and metastatic renal cell cancer and another trial is

evaluating use of IL15 after chemotherapy combined with the administration of patient-derived tumor-infiltrating lymphocytes (TIL) in metastatic melanoma [145]. None of the clinical trials yet employ IL15/IL15Rα complexes (discussed below).

IL15 activates important mechanisms of antitumor immunity, including development and activity of both NK cells and $CD8^+$ T cells and promoting development of memory T cells [146]. These functions overlap with the functions of IL2, with whom IL15 shares the IL2R/IL15Rβ and common gamma (γ_c) chain receptor subunits [145]. However, in many cancer relevant adaptive immune responses, IL2 and IL15 have distinct roles. Unlike IL2, IL15 does not induce regulatory T cell activity (Tregs) which attenuate immune response to cancer [147]. IL15, in contrast to IL2, inhibits activation-induced cell death (AICD) of $CD8^+$ effector T cells [148]. Application of high doses of IL2 is accompanied by severe toxicity [149], while preclinical studies have shown little vascular capillary leak with IL15 in contrast to IL2 [150].

IL15 belongs to the family of the common gamma chain (γ_c) or four-helix-bundle cytokines, which includes IL2, IL4, IL7, IL9, IL15, and IL21. Receptors for these cytokines share the common γ_c chain (CD132) and have unique α chains [146]. IL2R and IL15R have additional identical IL2/15Rβ chains (CD122), as mentioned previously. Although IL15 mRNA expression is widespread, IL15 protein is largely detectable only in monocytes/macrophages and dendritic cells. IL15 expression is tightly regulated at the post-transcriptional level, probably because of its potency as an inflammatory cytokine [145].

The unique α subunit of the IL15 receptor complex (IL15Rα) binds to IL15 with high affinity (Kd less than 10^{-11} M) and retains IL15 on the cell surface. In the physiological setting, IL15 is trans-presented by IL15Rα to the IL2/15Rβ-γ_c receptor component on nearby effector NK and T cells [151] [152]. IL15 can persist in this membrane-bound form for many days via endosomal recycling and this mechanism probably limits exposure of target cells to circulating IL15 [145]. IL15 signaling in lymphocytes activates the JAK1/JAK3 and STAT3/STAT5 pathways, Syk kinase and phospholipase C (PLC)γ, Lck kinase, and Shc resulting in the activation of PI3K/Akt and Ras/Raf/MAPK signaling cascades. These pathways lead to the subsequent expression of *Bcl2, Myc* and *Fos/Jun* and NF-κB activation [153].

Many approaches have been employed in an effort to increase the antitumor effects of IL15. Since IL15Rα may be considered a part of the active IL15 cytokine complex rather than part of the receptor, some of the improving approaches include pre-association of IL15 with IL15Rα [146]. Mutual stabilization of IL15 and IL15Rα leads to increases in production, stability, and tissue availability of bioactive IL15 *in vivo* [154]. Soluble IL15/IL15Rα complexes greatly enhanced IL15 half-life and bioavailability *in vivo* and dramatically reduced tumor burden in a model of B16-F10 melanoma [155] [156]. IL15 preassociated with a chimeric protein of the extracellular domain of murine IL15Rα and the Fc portion of human IgG1 also

increased antitumor activity against melanoma [157]. Importantly, this effect is preserved when the cytokine and the α receptor subunit are covalently joined and expressed as a single protein. Fusion protein of the N-terminal domain of IL15Rα and IL15 reduced tumor burden in the mouse B16F10 melanoma model and in an orthotopic human colon carcinoma model [158]. In Kranz laboratory the strategies of single chain TCR-mediated targeting of the IL15-IL15Rα fusion protein are currently explored [159]. These results hold significant importance for the use of IL15/IL15Rα complexes as a potential adjuvant or therapeutic.

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CHAPTER TWO

MYXOMA VIRUS EXPRESSING A FUSION PROTEIN OF INTERLEUKIN-15 (IL15) AND IL15 RECEPTOR ALPHA HAS ENHANCED ANTITUMOR ACTIVITY

2.1 Introduction

The oncolytic potential of many viruses, such as the poxviruses vaccinia virus and myxoma virus, initially suggested that they could be used as cancer therapy, but the efficacy of such viruses as a single agent *in vivo* has been limited [1]. Alternatively, the selectivity of such oncolytic and oncotropic viruses can be used to deliver cytokine genes to cancer cells [2][3]. One goal of this approach is to shift the immunosuppressive microenvironment found in many solid tumors to an environment that better favors the induction of antitumor immune responses.

Myxoma virus is an oncotropic poxvirus that has a particularly attractive safety profile. In the wild, the virus infects only rabbits and other related leporids, and is nonpathogenic in all other nonlagomorph animals tested [4]. Despite its lack of broad pathogenicity other than the rabbit, myxoma virus can replicate in diverse cultured cells from many species, including most human cancer cells, which are particularly permissive for the virus [5][6][7]. It also selectively infects tumors in human xenograft models [8][9][10][11] and primary mouse tumors [11][12][13]. It has recently been shown that myxoma virus can discriminate cancerous human myeloid cells from normal $CD34^+$ stem cells, which makes it a potential *ex vivo* purging agent for hematological malignancies [14][15].

Some oncotropic viruses tested in clinical trials have been modified to express an immunostimulatory cytokine, GM-CSF [16][17]. Although GM-CSF is a cytokine with potentially favorable antitumor activity, it can also stimulate suppressive components of the immune system [18]. Therefore, it is worth exploring other cytokine candidates to be delivered by a tumor-selective viral vector, particularly those that are known to be capable of activating non-responsive or anergic cytotoxic lymphocytes [19].

IL15 is a pro-inflammatory cytokine with significant potential for stimulating T lymphocytes and NK cells against cancer [20]. IL15 expression is tightly regulated at the post-transcriptional level, making IL15 protein largely detectable only in monocytes/macrophages and dendritic cells [21]. Co-expression of IL15 with the α subunit of IL15 receptor (IL15Rα) greatly enhances IL15 stability and function *in vivo* [22][23][24]. Since IL-15Rα may be considered a part of the active IL-15 cytokine complex rather than part of the receptor, pre-association of IL-15 with IL-15Rα generates a more potent ligand compared to the cytokine alone [25][26][27][28]. Recombinant myxoma viruses have previously been engineered to express tdTomato red fluorescent protein (vMyx-tdTr) and mouse interleukin-15 (vMyx-IL15-tdTr) [29]. Our previous studies have shown that these myxoma viruses (vMyx-tdTr and vMyx-IL15-tdTr) productively

infect cancer cells *in vitro*, but have limited effect on tumor progression of murine melanoma in immune competent mice *in vivo* [30][31]. In order to deliver the biologically potent form of IL15 with its IL15Rα component *in vivo*, we engineered a new recombinant myxoma virus (vMyx-IL15Rα-tdTr), which expresses IL15Rα-IL15 fusion protein, as well as tdTomato red fluorescent reporter protein.

In this study, we describe the therapeutic effects observed with the new recombinant virus in a mouse model of aggressive melanoma, B16-F10. *In vitro* testing of the virus showed that B16-F10 cells are permissive to the vMyx-IL15Rα-tdTr infection. Secretion of the IL15Rα-IL15 fusion protein was confirmed by ELISA and functional activity of the fusion was assessed by a proliferation assay on IL15-dependent CTLL-2 cells. In *in vivo* experiments, immunohistological analysis of the subcutaneous tumors showed dramatically increased infiltration of NK cells in vMyx-IL15Rα-tdTr treated tumors compared to controls in $RAG1^{-/-}$ mice. In immunocompetent C57BL/6 mice, vMyx-IL15Rα-tdTr increased infiltration by NK cells and $CD8^{+}$ T cells. $RAG1^{-/-}$ mice with subcutaneous B16-F10 tumors were treated with vMyx-IL15Rα-tdTr, resulting in a significant survival benefit for the treated group compared to the PBS control and the control viruses (vMyx-IL15-tdTr that expresses the native IL15 ligand and control vMyx-tdTr). Treatment of tumor-bearing C57BL/6 mice with vMyx-IL15Rα-tdTr resulted in longer survival than similarly treated $RAG1^{-/-}$ mice. Our results suggest that virally delivered IL15Rα-IL15 drives the recruitment of NK cells and T cells to the site of the tumor and that both the innate and adaptive components of the host immune system play a role in the antitumor effect.

2.2 Materials and methods

DNA constructs

pBluescript SK+ plasmid that served as a cloning backbone for the IL15Rα-IL15 – tdTomato expression cassette was obtained from ATCC (Manassas, VA). Whole viral DNA isolated from vMyx-tdTr [29] using the DNeasy Tissue Kit (Qiagen, Valencia, CA) was used as a template for obtaining the PCR fragment containing partial sequences of M135 and M136 genes. HindIII and BamHI cutting sites were introduced in this PCR reaction; primers for all PCR reactions were obtained from Integrated DNA Technologies (Coralville, IA) (Forward primer: 5'- CCA AAG CTT CAC CTG TGT ATG TT -3', Reverse primer: 5'- CCA GGA TCC ATA ACA CAC AGT TCG G -3'). PCR product from vMyx-tdTr was ligated into pBluescript using the T4 Ligase (Invitrogen, Carlsbad, CA) following sequential digestion with HindIII and BamHI (New England BioLabs, Ipswich, MA). IL15Rα-IL15 fusion protein contains codon-optimized sequence for the murine IL-15Rα sushi domain (amino acids 34-103 of Isoform 1, UniProt accession #Q60819), a linker with the sequence $GG(SGG)_6$ and murine IL-15; it was purchased from GenScript (Piscataway, NJ) [32]. Poxvirus vvSynE/L promoter, murine Ig κ-chain leader sequence (which directs the protein to the secretory pathway) as well as BspEI and NdeI cutting sites were added and His tag was eliminated from the original IL15Rα-IL15 sequence using forward primer: 5'- CGC AGC TCC GGA AAA AAT TGA AAT TTT ATT TTT TTT TTT TGG AAT ATA AAT AAG ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC ACC ACC TGC CCC CCC CCC GTG -3' and reverse primer: 5'- TCG CGC CAT ATG TTA TCA GCT GGT GTT GAT GAA CAT CTG CAC G -3'. The resulting IL15Rα-IL15 sequence was cloned into the earlier described pBluescipt construct using BspEI and NdeI (New England BioLabs, Ipswich, MA) and T4 Ligase (Invitrogen, Carlsbad, CA). Finally, tdTomato was cloned out of the plasmid provided by Dr. Brian Freeman (University of Illinois) using primers: forward 5'- GCA GTC GAC ATG GTG AGC AAG G – 3' and reverse: 5'- CCT GAA TTC TTA CTT GTA CAG CTC G – 3' and cloned into the existing pBluescript construct using SalI and EcoRI. Resulting plasmid, pBS-IL15Rα-IL15-tdTomatoRed (Figure 2.1) was used for creating vMyx-IL15Rα-tdTr.

Recombinant viruses

The Lausanne strain of myxoma virus (vMyx-Lau) was used to create recombinant virus expressing tandem dimer Tomato red fluorescent protein (vMyx-tdTr) with or without expression of interleukin-15 (vMyx-IL15-tdTr) by intergenic insertion of the gene cassettes between M135R and M136R of the myxoma virus genome as previously described [29]. Protein expression of IL15 by this recombinant virus is driven by a vaccinia virus late promoter (p11).

The recombinant virus expressing the IL15Rα-IL15 fusion protein (vMyx-IL15Rα-tdTr) was created by homologous recombination in RK-13 cells infected with wild type (WT) vMyx-Lau followed by transfection with the engineered recombination vector pBS-IL15Rα-IL15-tdTomatoRed (Figure 2.1). The recombination vector contains genes for the IL15Rα-IL15 fusion protein and tdTomato, both under control of the same synthetic vaccinia virus early/late promoter (vvSynE/L promoter). This expression cassette is flanked by M135 and M136 partial gene sequences for the purpose of being transfected into the WT myxoma virus genome between genes M135 and M136. Myxoma virus permissive RK-13 cells were infected with WT vMyx-Lau followed by cationic lipid transfection of the engineered recombination vector pBS with the IL15Rα-IL15-tdTr cassette. After plasmid recombination into the virus, recombinant virus expressing IL15Rα fusion protein was propagated and titrated by focus formation on RK-13 cells. Fluorescent virus foci were harvested, repropagated and titrated on RK-13 cells. This process was repeated three times to isolate a purified virus which contains two exogenous genes; IL-15Rα-IL15 fusion protein and tdTomato. Genomic structure of recombinant virus was confirmed by PCR sequencing.

Cell culture and reagents

Rabbit kidney epithelial (RK-13) cells were a gift from Dr. Richard Moyer (University of Florida, Gainesville, FL; originally from ATCC, Manassas, VA). RK-13 are grown at 37°C, 5% CO2, and 100% humidity in minimum essential medium with Earle's salts (Mediatech, Manassas, VA) supplemented with 2 mM glutamine, 50 U/mL penicillin G, 50 µg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (MEM-C), and 10% fetal bovine serum (FBS; HyClone, Logan, UT).

The murine melanoma cell line, B16-F10 was purchased from ATCC (Manassas, VA). The murine glioma cell line GL261 was obtained from the National Cancer Institute-Frederick Cancer Research Tumor Repository (Frederick, MD). B16.SIY was derived from B16-F10 cells retrovirally transduced to express green fluorescent protein (GFP) as a fusion protein with SIYRYYGL (SIY) [33,34] and was a gift from Dr. Thomas Gajewski (University of Chicago, Chicago, IL).

Cancer cell lines were cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium containing 5 mM HEPES, 1.3 mM L-glutamine, 50 µM 2-ME, penicillin, streptomycin and 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Cytotoxic T Lymphocyte Line 2 (CTLL-2) cytokine-dependent murine T cell line (ATCC, Manassas, VA) was cultured in complete RPMI 1640 medium additionally supplemented with 10% T-Stim (culture supernatant from rat T cells stimulated with ConA from BD Biosciences, San Jose, CA).

Viral growth curves

Tested cell lines were plated into 6-well cell culture plates (Nunc, Roskilde, Denmark) and grown in MEM-C with 10% FBS until they reached 90-95% confluency. For multi-step growth curves cells were inoculated with vMyx-IL15Rα-tdTr or vMyx-tdTr diluted in 400µL MEM-C at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell. Inoculated cells were incubated at 37°C and 5% CO_2 for 1h, rocking every 15 min. Next, virus was removed, cell monolayers were washed with phosphate-buffered saline (PBS), and MEM-C with 10% FBS was added to each well. Inoculations of each cell line were performed in triplicate. At 0, 4, 8, 12, 24 and 48 h post-inoculation, cells were dislodged by scraping into media. Cells were collected by centrifugation at 500xg for 5 min. Next, each supernatant was removed, and the cellular pellet was resuspended in 0.5 ml PBS and stored at -80°C. Prior to titering the virus, cells were disrupted by three freeze/thaw cycles and sonication in order to release the virus from the cells. Samples were titered in duplicate. Titering was performed by plating 10-fold serial dilutions of the samples in MEM-C onto RK-13 monolayers in 6-well or 24-well culture plates. The inoculated cells were incubated for 1h at 37°C and 5% CO_2, then the inoculum was removed and an overlay consisting of equal amounts of 1% agarose (Lonza, Rockland, ME) and 2×MEM-C with 20% FBS was added on RK-13 cells. Viral plaques were visualized as small white foci (red foci under fluorescent light) and counted at 6-7 days post-inoculation (dpi).

ELISA analysis of the IL15Rα-IL15 fusion protein

RK-13 cells were plated in 6-well culture plates and upon reaching 90-95% confluency they were inoculated with vMyx-IL15Rα-tdTr or vMyx-tdTr diluted in 400µL MEM-C at MOI of 5 PFU/cell. After 1h incubation at 37°C and 5% CO_2, inoculum was replaced with MEM-C with 10% FBS. At different time points post-inoculation, both cell supernatant and cellular extract were collected. Supernatants were centrifuged briefly to remove cellular debris and clarified supernatants were transferred to new tubes and stored at -80C. The remaining cellular monolayer was detached from the well by scraping cells into 1 ml PBS. Cells were collected, pelleted by brief centrifugation (1,300 rpm x 1 min), and cellular pellets were resuspended in Cytoplasmic Extract (CE) buffer supplemented with HALT protease inhibitor cocktail (Thermo Fisher, Rockford, IL). Samples were incubated for 5 min at 4°C and centrifuged at 1,300 rpm for 1 min. Supernatants were moved to new tubes are stored at -80°C.

For IL15Rα-IL15 detection by ELISA, the Mouse IL-15/IL-15R Complex ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA) was used. All samples were 10-fold serially diluted and each dilution was done in duplicate. Each kit included a purified protein standard which was used to establish a standard

curve. An ELx800 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT) was used to detect absorbance at 450 nm.

Western Blot

For Western Blot analysis, samples were transferred from the SDS-PAGE gels (precast Mini-PROTEAN TGX gels by Bio-Rad, Hercules, CA) to Millipore Immobilon-P Transfer membrane (Millipore, Billerica, MA) using the semi-dry transfer method (Trans-Blot® SD Semi-Dry Transfer Cell by Bio-Rad, Hercules, CA). Membranes were blocked in 4% skim milk in PBS. Antibodies were diluted in PBST/0.5% BSA. For IL15 detection, anti-IL15-biotin antibody from eBioscience (San Diego, CA) was used, coupled with IRDye 800CW Streptavidin (SA-IRD800) from LI-COR (LI-COR Biosciences, US). For IL-15Rα detection, Mouse IL15 R alpha Affinity Purified Polyclonal Ab (R&D Systems, Minneapolis, MN) was used as a primary antibody, Rabbit polyclonal Antibody to Goat IgG - H&L (Biotin) (Abcam, Cambridge, MA) was used as a secondary antibody and finalized with SA-IRD800 as described previously. Blots were visualized on the Odyssey Infrared Imaging System (LI-COR Biosciences, US). Molecular weight markers used were Broad Range Prestained SDS-PAGE Standards from Bio-Rad (Hercules, CA). In each repeat of the experiment two separate, identically loaded SDS-PAGE gels were run, samples from each gel were transferred to a corresponding membrane, one of which was stained for IL15, and the other for IL15Rα.

CTLL-2 cell proliferation assay

MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and detergent were purchased from ATCC (Manassas, VA). Confluent RK-13 cells in 6-well plates were infected with vMyx-IL15Rα-tdTr or vMyx-tdTr at MOI=5. At 24h and 48 h p.i., cell-free media was collected and stored at -80°C. CTLL-2 cells were propagated overnight at 37°C, 5% CO_2 in complete RPMI media with no added cytokines. Next, CTLL-2 cells were collected by centrifugation and resuspended at 50,000 cells per well in a 96-well plate in 100 µL complete RPMI containing either 10^{-9}M IL-2, 10^{-9}M TCR-IL15Rα (fusion of the m33 TCR with IL15Rα-IL15, "m33-superfusion" [32]), vMyx-tdTr or vMyx-IL15Rα-tdTr infected cell media. The cells were cultured for 48 h, and then 10 µL MTT was added per well, and the cells were incubated at 37°C, 5% CO_2 for three more hours, and then 100 µL per well detergent was added, and the plate was incubated at room temperature overnight. To estimate CTLL-2 cell proliferation in different conditions, absorbance at 570 nm in each well was read using an EL_x800 universal microplate reader (Bio-Tek Instruments, Winooski, VT).

Animals

C57BL/6 and C57BL/6 RAG1$^{-/-}$ mice originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were maintained as colonies and housed in the animal facilities at the University of Illinois. Mice were used in experiments when they were 2-5 months old. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign (PHS Assurance A3118-01, AAALAC, International Accreditation #00766). Anesthesia was used during tumor cell and virus injections, and all efforts were made to minimize suffering.

Subcutaneous tumor establishment and treatment

B16-F10 melanoma cells were harvested and washed twice with Hanks Balanced Salt Solution (HBSS, Cellgro Mediatech Inc., Manassas, VA). Prior to all procedures, mice were anesthetized by isoflurane (Aerrane, Baxter, Deerfield, IL) inhalation using the classic vaporizer unit by E-Z Systems (Palmer, PA). Shaved mice received 1x 10^6 tumor cells in 100µl HBSS subcutaneously into the right flank. After 7 days, when tumors usually reach a volume of approximately 100 mm^3, mice were assigned to treatment groups and received an intratumoral (i.t.) injection of virus. At this time, tumors were directly injected with 2.6×10^7 PFU of sucrose-pad purified vMyx-IL15α-tdTr, vMyx-IL-15-tdTr or vMyx-tdTr that was in a final volume of 50 µl. A separate set of mice received 50 ul PBS i.t. An additional i.t. inoculation of each virus (2.6×10^7 PFU) occurred 3 days later (day 10 post-implantation). For those tumors that were large, the inoculum was injected into at least three different sites to introduce the virus throughout the mass. Prior to all repeated tumor injections with either virus or PBS, animals were anesthetized by isoflurane inhalation as described earlier. All animals were single housed upon tumor cell implantation and during all subsequent experimental manipulations.

Tumor growth was monitored by measuring tumor length, width and height with a caliper. Tumor volume was calculated as ((length) x (width) x (height))/2. Mice were monitored daily. Mice were humanely euthanized when tumors reached the volume of 3000 mm^3, or showed lethargy or signs of pain, or when animal lost 25% baseline body weight. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. In some experiments, samples from some mice were collected 3 days after final virus treatment for histological analysis, and other mice in each treatment group were monitored until they reached a criterion for euthanasia.

Tissue sections and immunostaining

After the mice were euthanized, their subcutaneous tumors were snap-frozen in OCT medium for cryosectioning and immunostaining. Eight µm cryosections were taken. Primary antibodies used for

staining were: 4D11 (rat anti-Ly-49G2, BD Pharmingen, San Jose, CA), rabbit anti-CD3 (Abcam, Cambridge, MA), rat anti-CD8 (eBioscience, San Diego, CA), rat anti-CD4 (Abcam, Cambridge, MA). Secondary antibodies used: biotin rabbit anti-rat and biotin goat anti-rabbit (Vector, Burlingame, CA). For immunostaining, slides were fixed in cold 95% ethanol and blocked with Superblock (Thermo Scientific, Rockford IL). Sections were then incubated with a primary antibody in PBS + 20% glycerol (PBSG) overnight, washed with PBS + 0.1% Tween-20 (PBST), and incubated with biotinylated secondary antibody in PBST for 4 h. Slides were washed and incubated with streptavidin-Alexa Fluor 594 (Invitrogen, Carlsbad, CA) or streptavidin-Alexa Fluor 488 (Jackson ImmunoResearch, West Grove, PA) and DAPI (Invitrogen, Carlsbad, CA). Control slides omitting the primary antibody were negative for Alexa Fluor 594 or Alexa Fluor 488. Images were obtained with an Olympus BX-51 microscope at 20x magnification.

Data Analysis

GraphPad Prism software (La Jolla, CA) was used for all statistical analyses and graph presentation. Survival data were recorded from the time of the tumor cell implantation until euthanasia and were plotted using a Kaplan-Meier curve. Survival treatment groups were compared with a Log-rank (Mantel-Cox) test. Virus growth curves were analyzed by two-way ANOVA, ELISA data were analyzed by t-test at corresponding time points, the bioassay of IL-15 activity was analyzed by one-way ANOVA, and histological cell counts were analyzed by one-way ANOVA with Bonferroni planned comparisons. Significance was considered $p < 0.05$.

2.3 Results

Murine melanoma and glioma cell lines are permissive for recombinant myxoma virus infection

Prior to testing the therapeutic capacity of the recombinant IL15Rα-IL15 virus (vMyx-IL15Rα-tdTr), we tested its capacity to infect relevant murine cancer cell lines *in vitro.* Multi-step growth curves of vMyx-IL15Rα-tdTr and the previously characterized vMyx-tdTr control virus [29], showed similar patterns of permissiveness in various cell lines that were tested (Figure 2.2). Melanoma cell lines (B16-F10 and B16.SIY) were as permissive as the positive control rabbit cell line RK-13. For all three cell lines, infectious viral particles were formed by 12 h post infection, and maximal viral titer was typically obtained at the 48 h time point. vMyx-IL15Rα-tdTr and vMyx-tdTr showed a different growth phenotype in the glioma cell line (GL261) and produced lower viral titers (Figure 2.2B). Based on the observation that the insertion of the IL15Rα-IL15 gene did not impact the infectivity of the virus, vMyx-IL15Rα-tdTr was considered a useful system to deliver functional IL15Rα-IL15 fusion protein to B16 tumors.

IL15Rα-IL15 fusion protein is expressed and secreted *in vitro* by virus infected cells

To determine if cells infected with vMyx-IL15Rα-tdTr were capable of secreting IL15Rα-IL15 fusion protein, we examined the culture media and cell extracts of infected cells using an ELISA specific for the IL15/IL15R complex. IL15Rα-IL15 was detected in both supernatants and cell extracts of vMyx-IL15Rα-tdTr infected RK-13 cells (MOI=5) as compared to the control non-cytokine expressing virus vMyx-tdTr (Figure 2.3A). The peak of cell-associated expression of the fusion protein occurred at 12 h post-infection (mean value of 73 ng/ml), while secreted levels peaked at 48 h post-infection (mean value of 663 ng/ml). IL15Rα-IL15 was present in ten-fold higher levels in cellular supernatants versus cell-associated. As would be expected, cells infected with non-cytokine expressing virus (vMyx-tdTr) did not show measurable levels of IL15Rα-IL15. The presence of IL15 and IL15Rα domains was also confirmed by Western blot of supernatants and cell extracts (Figure 2.3B). These findings showed that the IL15Rα-IL15 gene that was stably inserted into myxoma virus was expressed and that the fusion protein was secreted from infected cells at high levels (over 500 ng/ml).

IL15Rα-IL15 fusion protein secreted by vMyx-IL15Rα-tdTr infected cells is functionally active

Functional activity of the IL15Rα-IL15 fusion protein in the supernatants of virus-infected cells was assayed by its ability to induce proliferation of cytokine-dependent CTLL-2 cells (Figure 2.4). CTLL-2 is a clone of T cells that requires IL-2 or other growth-promoting cytokines for proliferation [35]. MTT cell proliferation assays showed that CTLL-2 cells cultured in medium supplemented with supernatants of vMyx-IL15Rα-tdTr infected cells proliferated to the similar extent as CTLL-2 cells incubated with

recombinant IL-2 (10nM) or a purified fusion protein of IL15Rα-IL15 and a single-chain TCR m33 (10nM) [32]. In contrast, CTLL-2 cells cultured with supernatant of the control virus vMyx-tdTr were not stimulated to proliferate.

Treatment with IL15Rα-IL15 fusion protein expressing myxoma virus results in increased presence of NK cells in tumors of RAG1$^{-/-}$ mice

We next tested whether this new recombinant virus would affect cellular immune responses *in vivo*. Because NK cells are responsive to IL15 [36], we investigated whether treatment with vMyx-IL15Rα-tdTr was associated with the presence of NK cells in subcutaneous tumors of RAG1$^{-/-}$ mice, which have NK cells but no T or B cells. Accordingly, RAG1$^{-/-}$ mice bearing established subcutaneous B16-F10 tumors were injected intratumorally (i.t.) with vMyx-IL15Rα-tdTr, vMyx-tdTr or PBS on days 7 and 10 post tumor cell injection. Tumor sections of mice treated with the virus expressing IL15Rα-IL15 fusion protein showed dramatic and significant increase in numbers of infiltrating NK cells, compared to vMyx-tdTr and PBS treated tumor (Figure 2.5). This evidence suggests a role of NK cells as a component of the host immune system that may contribute to an antitumor effect.

vMyx-IL15Rα-tdTr treatment enhances both NK cell and T cell recruitment to subcutaneous tumors in C57BL/6 mice

To determine the effects of the virus in fully immunocompetent animals, we repeated the experiment using C57BL/6 mice. C57BL/6 mice with subcutaneous B16-F10 tumors were injected intratumorally with vMyx-IL15Rα-tdTr, vMyx-tdTr or PBS on days 7 and 10 post tumor cell injection. Similar to the effect observed in RAG1$^{-/-}$ mice, C57BL/6 mice treated with vMyx-IL15Rα-tdTr also had significant intra-tumor infiltration of NK cells, compared to both tdTomato expressing virus and PBS treatment (Figure 2.6A, B). In addition, T cell infiltration mirrored that of NK cells (Figure 2.6C, D). Analysis of T cell subsets in this response revealed that most tumor infiltrating T cells were CD8^{+}, although CD4^{+} cells were also elevated in vMyx-IL15Rα-tdTr treated tumors compared to controls (Figure 2.7).

Mice bearing subcutaneous melanoma tumors live longer when treated with IL15Rα-IL15 fusion protein-expressing virus compared to control viruses

For survival experiments, mice with established B16-F10 s.c. tumors were treated the same way as described for histological analysis (intratumoral virus treatment on days 7 and 10 post tumor cell injection) and were monitored for survival. For RAG1$^{-/-}$ mice, treatment groups were vMyx-IL15Rα-tdTr, vMyx-IL15-tdTr, vMyx-tdTr and PBS. Without any treatment (PBS), B16-F10 grows as an exceptionally aggressive tumor, with a median survival of 17 days. A small survival benefit was observed in the

tdTomato-only expressing virus group, similar to values obtained in a slightly different experimental setting [30]. Addition of the IL15 alone to the virus construct did not result in any improvement above this survival in RAG1$^{-/-}$ mice. However, addition of the IL15Rα-IL15 fusion protein improved therapeutic efficacy of myxoma virus compared to the other virus controls, including myxoma virus that expressed only the native IL15 domain (Figure 2.8). vMyx-IL15Rα-tdTr treatment resulted in tumor stabilization in the majority of animals until day 20, while mice given other treatments were succumbing to tumors at this point (Figure 2.8A). For C57BL/6 mice, treatment groups were vMyx-IL15Rα-tdTr, vMyx-tdTr and PBS. The antitumor effect of vMyx-IL15Rα-tdTr in immunocompetent animals showed the same pattern as in RAG1$^{-/-}$ mice, with overall longer median survival in corresponding groups (Figure 2.9). The effect on both strains is especially notable given that the time of treatment was when the tumors were already established and at the start of their aggressive growth phase.

2.4 Discussion

IL15 has been proposed as a useful cytokine for immunotherapy for cancer, and the complexing of IL15 with its receptor alpha component has been shown to enhance its biological activity. We therefore modified a viral system to deliver the fusion protein of IL15Rα-IL15, employing a myxoma virus vector with a strong safety profile. We confirmed that the vMyx-IL15Rα-tdTr virus has the same ability to infect melanoma cells as the previously characterized vMyx-tdTr control virus, and that it secretes biologically active IL15Rα-IL15.

IL15Rα-IL15 could potentially be delivered to tumors by a variety of means. For example, Bessard et al. delivered an IL15Rα-IL15 fusion protein by three systemic injections, prolonging the survival with a B16-F10 model for 7 days [28], and Dubois et al. injected IL15 preassociated with IL15RαFc, repeated as many as nine times, prolonging survival of B16-F10 bearing mice for 5 days [24]. In comparison, in the present study two injections of vMyx-IL15Rα-tdTr resulted in a prolongation of survival of 12 days in $RAG1^{-/-}$ mice and 20 days in C57BL/6 mice. Delivery by a viral vector results in secretion of virally encoded proteins peaking at 48-72 h and persisting for up to a week [31], so most likely fewer treatments would be needed to maintain the presence of the cytokine in the tumor environment.

In the survival experiments in $RAG1^{-/-}$ mice we compared effects of vMyx-IL15Rα-tdTr with vMyx-IL15-tdTr [29] (virus expressing IL15 but without the IL15Rα fusion component) as well as non-cytokine expressing vMyx-tdTr. Based on recent literature [23][37][38][39][40], adding the IL15Rα significantly improves IL15 effects compared to the cytokine itself. This was confirmed in our experimental setting: IL15-only expressing virus, consistent with published data [30], showed therapeutic effect against murine melanoma tumors in the RAG1-knockout background indistinguishable from vMyx-tdTr. Hence, for most of our other studies we compared the novel recombinant virus with the variant that was closer to wild type, expressing only the fluorescent protein.

Both NK cells and $CD8^+$ T cells responded to vMyx-IL15Rα-tdTr. In $RAG1^{-/-}$ mice, histological analysis revealed robust NK cell accumulation in the tumors of the treated animals. Previous studies have done depletion of NK cells prior to treatment to show that NK cells contribute to the antitumor effect of IL15/IL15Rα [28][41]. In some models, the effect of IL15Rα-IL15 is more dependent on $CD8^+$ T cells [27]. In immunocompetent C57BL/6 mice, both NK cells and $CD8^+$ T cells heavily infiltrated the tumors following vMyx-IL15Rα-tdTr treatment. Consistent with the idea that both cell types play a role in the effects of vMyx-IL15Rα-tdTr, treated C57BL/6 mice survived longer than treated $RAG1^{-/-}$ mice (43 days versus 29 days, $p < 0.05$).

Elpek et al. observed that sustained activation of NK cells by IL15/IL15Rα treatment (5 injections over 2 weeks) can lead to functional exhaustion of effector functions of NK cells [42]. Viral delivery by myxoma virus produces IL15Rα-IL15 secretion that is intermediate between a rapidly cleared systemic injection and chronic exposure. Future studies could determine an optimal interval for repetitive treatments to minimize NK cell exhaustion.

In addition to the delivery of IL15Rα-IL15, the myxoma construct itself may contribute to an enhanced immune response. Previously we demonstrated the feasibility of combining adoptive T cell therapy with concurrent administration of an oncolytic virus [31]. There are at least three potential mechanisms by which myxoma virus could kill susceptible tumor cells: First, virus can directly kill tumor cells by viral oncolysis; second, local production of antitumor cytokines caused by viral infection can lead to recruitment and activation of immune cells that better recognize and kill tumor cells; third, killed cancer cells can be a more potent source of cross-presented tumor peptides by tumor stroma to further enhance the acquired antitumor immune response [43]. Manipulation of tumor microenvironment is an important strategy to improve adoptive T cell therapy and eliminate occurrence of antigen loss variants (ALV), cells that lose the T cell reactive epitope and eventually lead to tumor outgrowth [31]. We hypothesized that delivery of a highly functional and potent IL15Rα-IL15 cytokine, especially in the context of viral infection, would provide a necessary boost to immune cells in driving their functional antitumor activities. Potential combination therapy along with the immunomodulating activities of anti-PD1/PDL1 antibodies, might provide an even more robust initial response and elimination of ALVs [44][45][27].

In summary, the use of delivery systems such as vMyx-IL15Rα-tdTr, and related genetically modified viruses, has the potential to improve clinical outcomes of cancer therapy.

2.5 References

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2.6 Figures

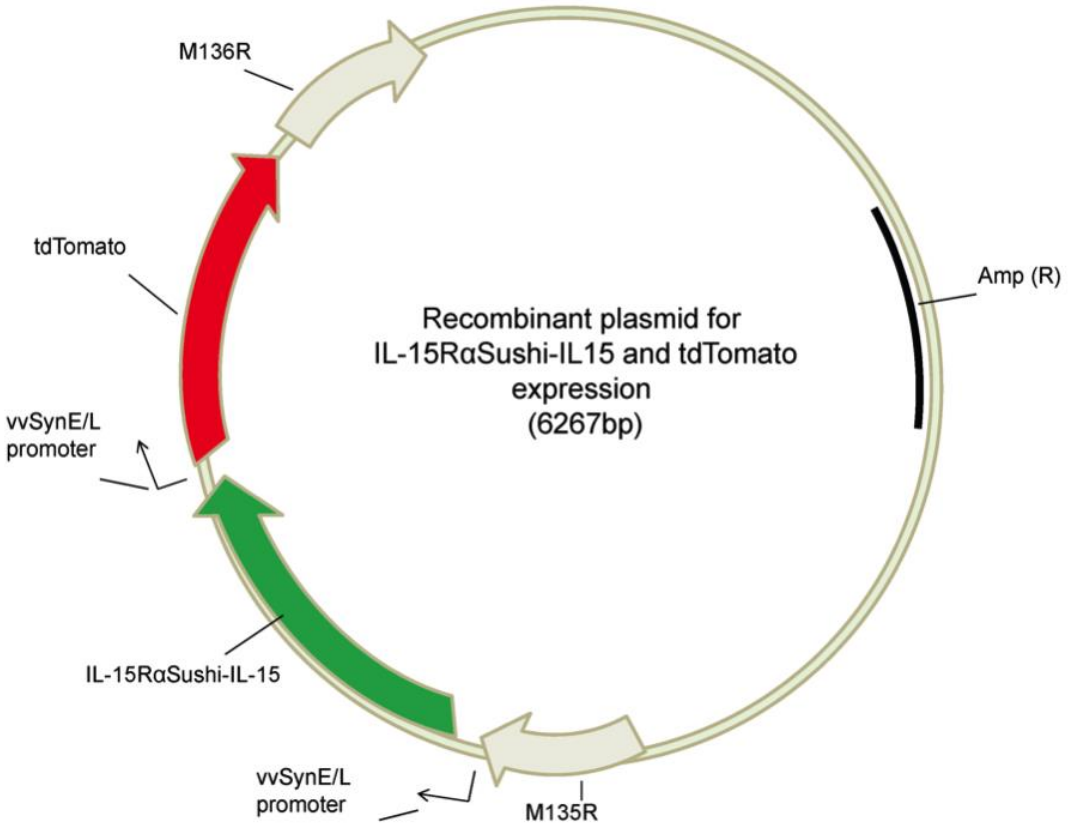

Figure 2.1. Recombinant plasmid for modifying WT myxoma virus and generating vMyx-IL15Rα-tdTr. Plasmid pBS-IL15Rα-IL15-tdTomatoRed (6267bp) is based on the pBluescript backbone on which M135 and M136 partial viral gene sequences are flanking genes for IL15Rα-IL15 fusion protein and tdTomato red fluorescent protein, both under control of vvSynE/L viral promoters. This expression cassette is flanked by partial viral gene sequences for the purpose of being transfected into the WT vMyx-Lau virus genome between genes M135 and M136.

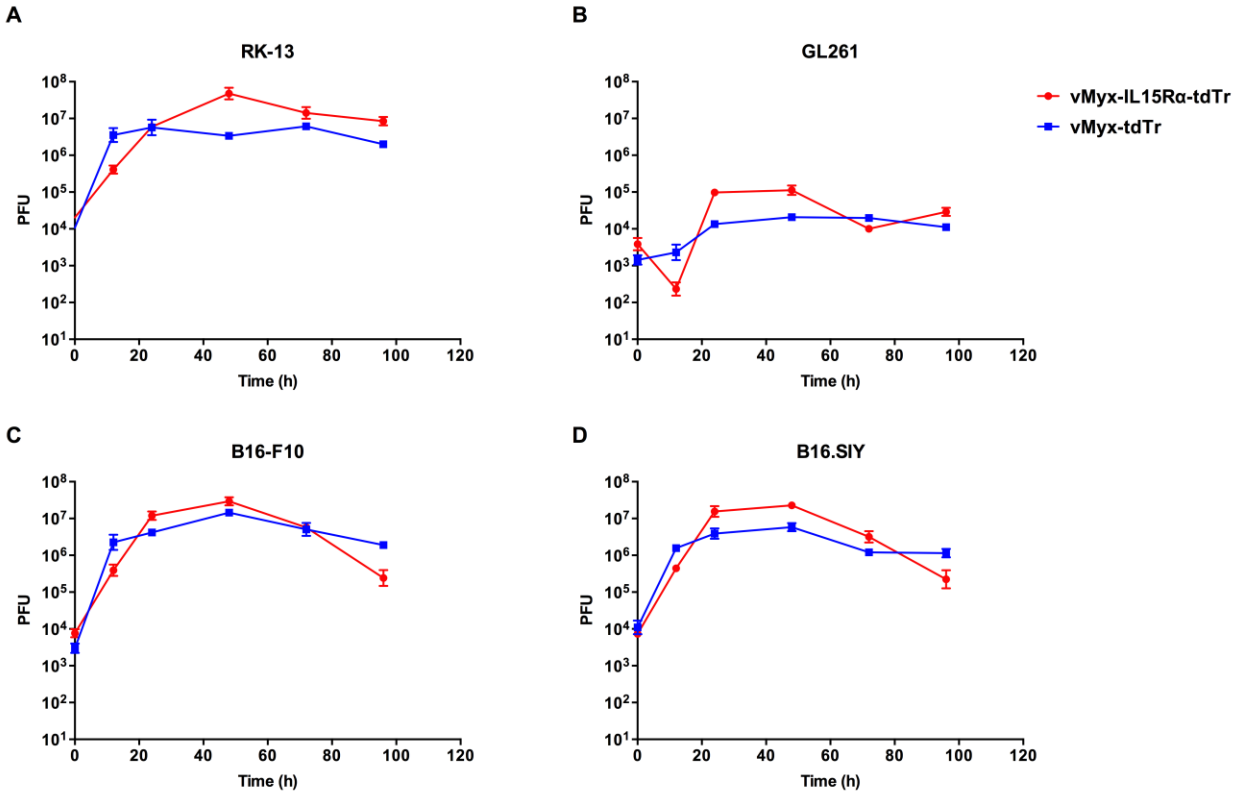

Figure 2.2. Melanoma and glioma cell lines are permissive to recombinant myxoma virus infection. Cell lines (A) RK-13, (B) GL261, (C) B16-F10, (D) B16.SIY, were infected with either vMyx-tdTr or vMyx-IL15Rα-tdTr at a multiplicity of infection (MOI) of 0.1 to obtain multi-step growth curves. At 0, 12, 24, 48, 72 or 96 h post-infection (p.i.), cells were harvested and lysed, and the viral titer was determined by titration on RK-13 cells. Error bars represent SEM from 3 replicates for each cell line. There was a significant effect of time for each of the cell lines ($p < 0.001$).

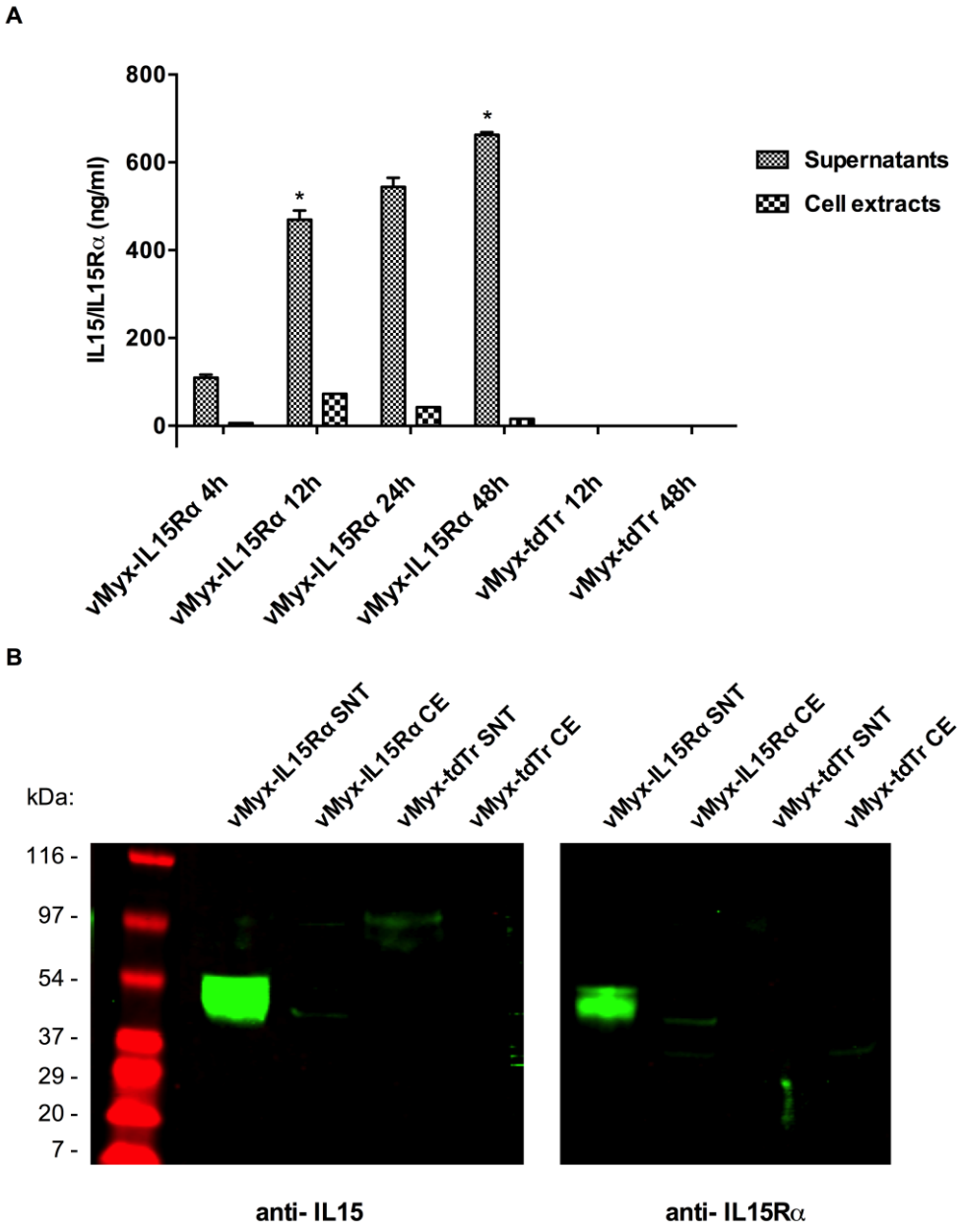

Figure 2.3. IL15Rα-IL15 fusion protein is present in the supernatants and extracts of cells infected with vMyx-IL15Rα-tdTr. Confluent RK-13 cells in 6-well plates were infected with vMyx-IL15Rα-tdTr or vMyx-tdTr at MOI=5. At indicated times post-infection, media was collected and cells were scraped, lysed and cytoplasmic extract was harvested. (A) ELISA. Mean ELISA values with SEM for triplicates of the same condition are presented, and the experiment was repeated with similar results. There was a significant increase in IL15Rα-IL15 fusion protein in supernatants of vMyx-IL15Rα-tdTr treated cells compared to vMyx-tdTr treated ones at corresponding timepoints (* - $p < 0.05$). (B) Western Blot. At 48 h post-infection, membranes blotted with supernatants and cell extracts of virus infected cells were stained for IL15 (left panel) or IL15Rα (right panel). Experiment was repeated five times with similar results. (SNT – supernatant, CE – cell extract)

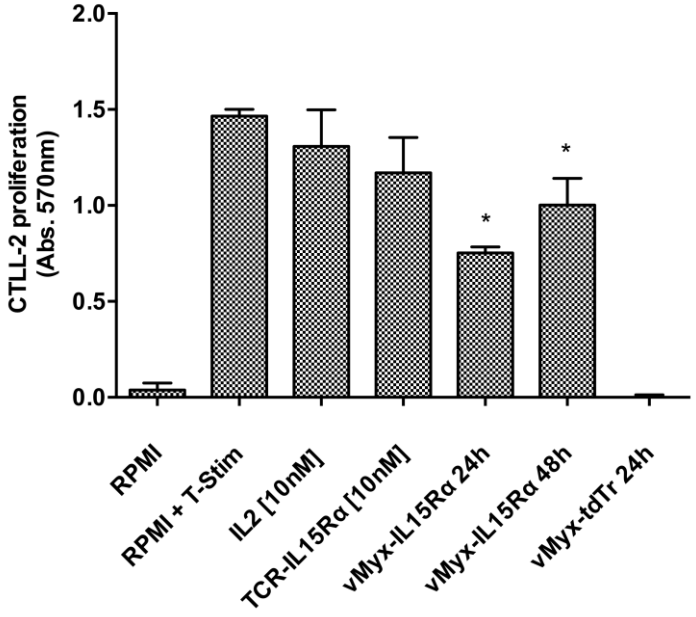

Figure 2.4. IL15Rα-IL15 fusion protein secreted by virus-infected RK-13 cells is functionally active. Confluent RK-13 cells in 6-well plates were infected with vMyx-IL15Rα-tdTr or vMyx-tdTr at MOI=5. At 24 h and 48 h p.i., cell media were collected. CTLL-2 cells were incubated with un-supplemented media (RPMI), three positive controls (media supplemented with 10% T-Stim (RPMI + T-Stim), 10^{-9}M IL-2 or 10^{-9}M TCR-IL15-IL15Rα fusion protein (TCR-IL15Rα)), or supernatants from cells infected with vMyx-tdTr or vMyx-IL15Rα-tdTr. CTLL-2 cell proliferation was analyzed by the MTT assay. Experiments were performed in triplicate for each treatment, and mean values with SEM are presented. Positive controls and supernatants from RK-13 cells infected with vMyx-IL15Rα-tdTr (marked by *) showed significant functional IL-15 activity, but supernatant from vMyx-tdTr infected RK-13 cells did not.

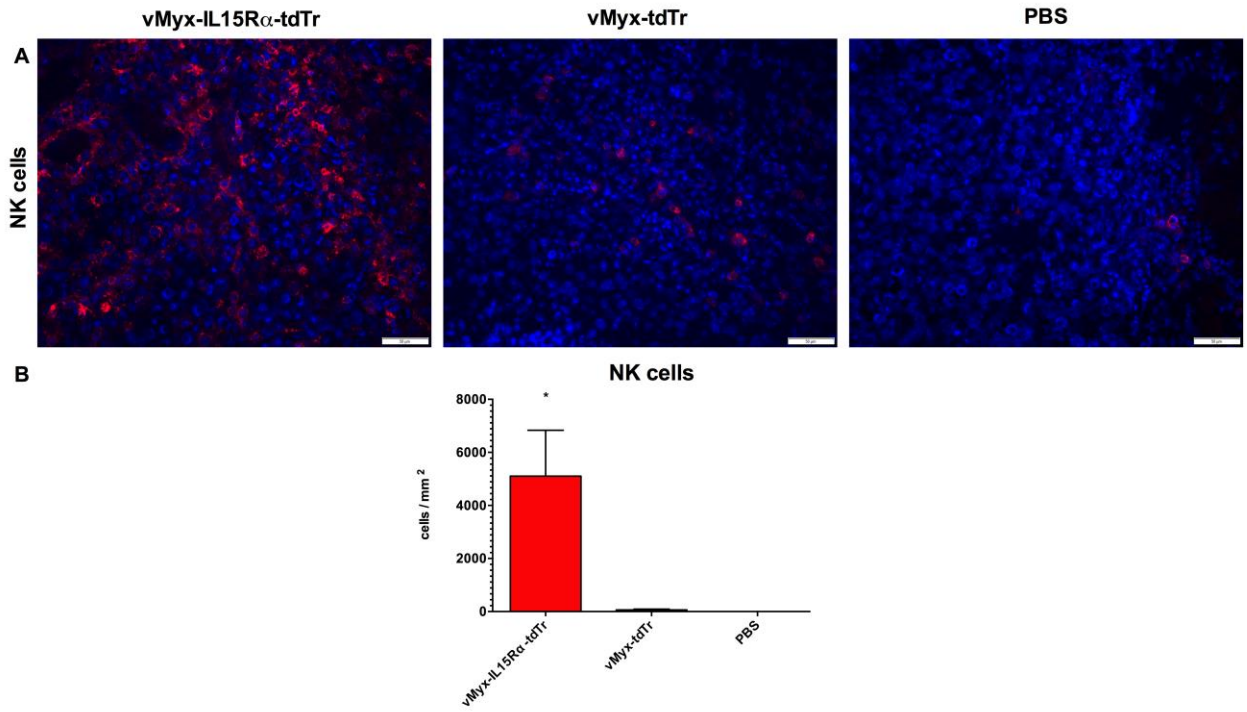

Figure 2.5. NK cell infiltration of subcutaneous B16-F10 tumors 3 days after intratumoral virus treatment in RAG1$^{-/-}$ mice. RAG1$^{-/-}$ mice (n = 3 per group) were implanted with unilateral subcutaneous B16-F10 tumor cells. The first dose of the virus (2.6×10^7 PFU i.t.) was given on day 7 (when tumors reached approximately 100mm^3) and the second dose was given on day 10. Treatment groups are: 1. vMyx-IL15Rα-tdTr 2. vMyx-tdTr 3. PBS. Mice were euthanized 3 days after the final virus treatment and tumor sections were analyzed for presence of NK cells by immunostaining for Ly-49G2 (4D11 antibody). Representative tumor sections are shown. (A) Staining for NK cells in tumors. Red – 4D11-positive stain, Blue – DAPI. Scale bar = 50 micrometers. (B) Estimated number of NK cells per square millimeter of a tumor section for each condition. Presented values are mean cell count in tumors from three mice, with SEM. One-way ANOVA showed significant increase in NK cell accumulation in vMyx-IL15Rα-tdTr treated tumors compared to both vMyx-tdTr and PBS treatments (* - $p < 0.05$).

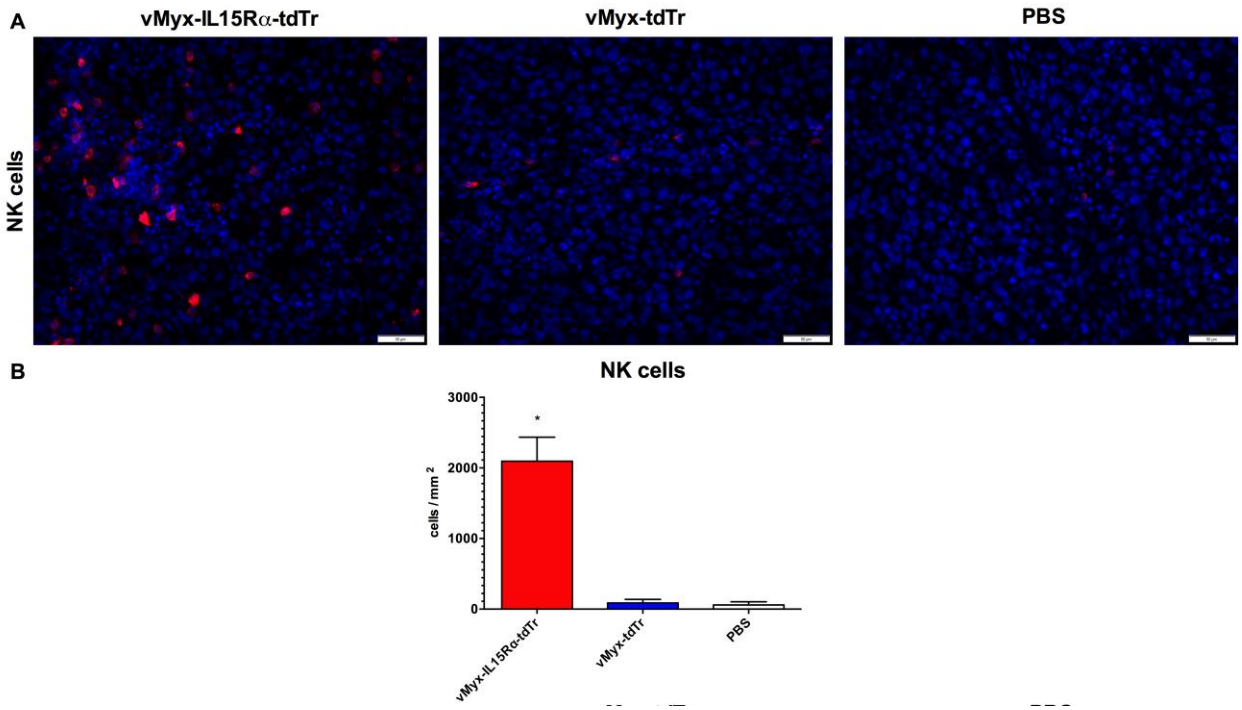

Figure 2.6. NK and T cell infiltration of subcutaneous B16-F10 tumors 3 days after intratumoral virus treatment in C57BL/6 mice. C57BL/6 mice (n = 3 per group) were implanted with unilateral subcutaneous B16-F10 tumor cells. The first dose of the virus (2.6×10^7 PFU i.t.) was given on day 7 (when tumors reached approximately $100mm^3$) and the second dose was given on day 10. Treatment groups are: 1. vMyx-IL15Rα-tdTr, 2. vMyx-tdTr, 3. PBS. Mice were euthanized 3 days after the final virus treatment and tumor sections were analyzed for presence of NK cells and T cells by immunostaining for Ly-49G2 (4D11 antibody) and CD3, respectively. Representative tumor sections are shown. (A) Staining for NK cells in tumors. Red – 4D11-positive stain, Blue – DAPI. Scale bar = 50 micrometers. (B) Estimated number of NK cells per square millimeter of a tumor section for each condition, mean values and SEM from 3 mice per group. One-way ANOVA showed significant increase in NK cell accumulation in vMyx-IL15Rα-tdTr treated tumors compared to both vMyx-tdTr and PBS treatments (* - $p < 0.05$). (C) Staining for T cells in tumors. Green – CD3-positive cells, Blue – DAPI. Scale bar = 50 micrometers. (C) Estimated number of T cells per square millimeter of a tumor section for each condition, mean values and SEM from 3 mice per group. One-way ANOVA showed significant increase in T cell accumulation in vMyx-IL15Rα-tdTr treated tumors compared to both vMyx-tdTr and PBS treatments (* - $p < 0.05$).

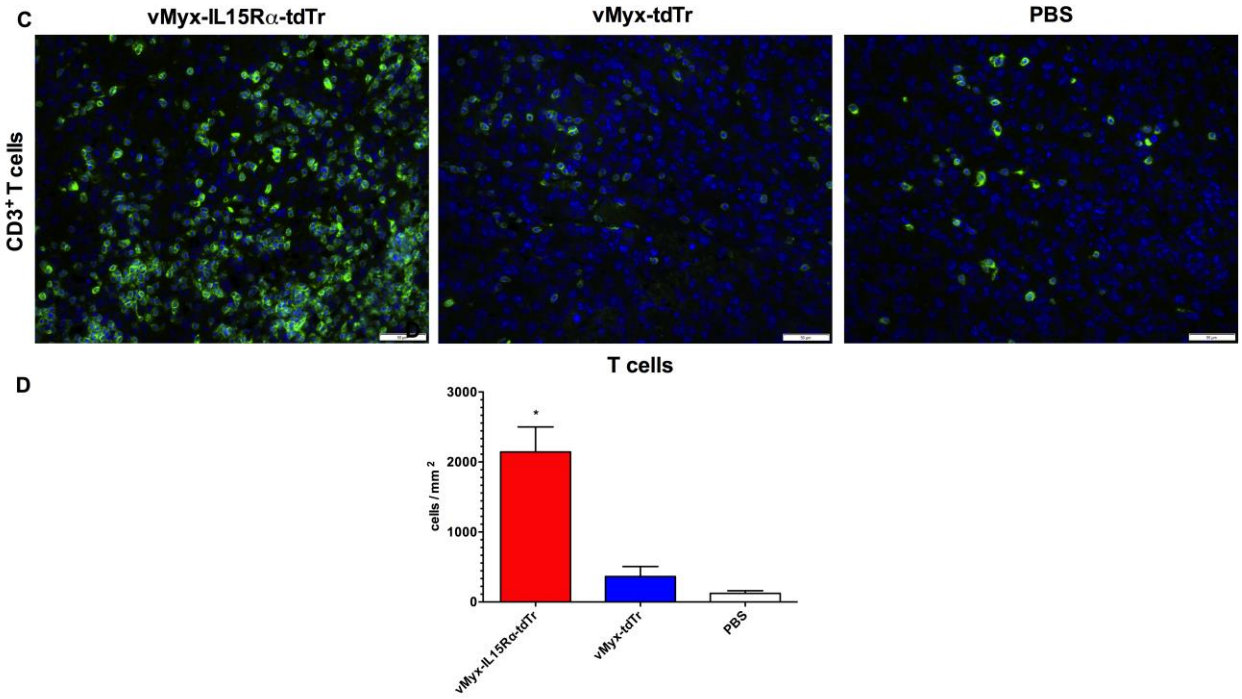

Figure 2.6 (cont'd). NK and T cell infiltration of subcutaneous B16-F10 tumors 3 days after intratumoral virus treatment in C57BL/6 mice.

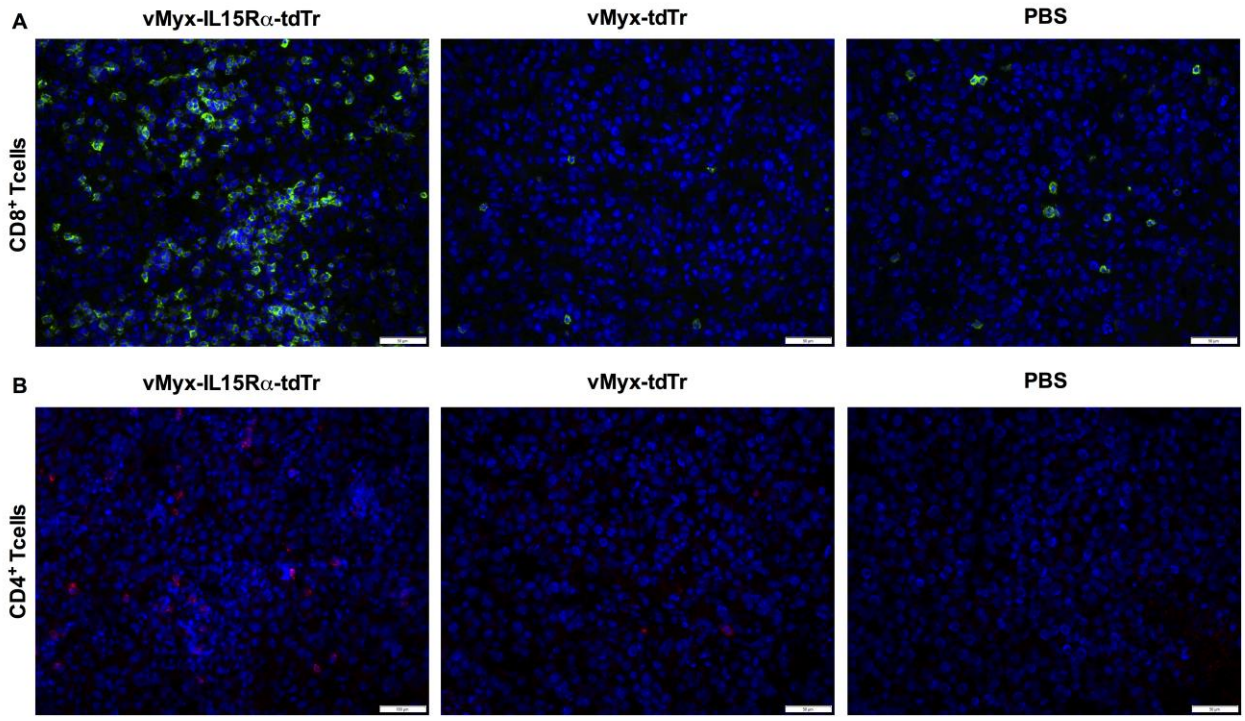

Figure 2.7. Analysis of subsets of T cells infiltrating subcutaneous B16-F10 tumors 3 days after intratumoral virus treatment in C57BL/6 mice. C57BL/6 mice (n = 3 per group) were implanted with unilateral subcutaneous B16-F10 tumor cells. The first dose of the virus (2.6×10^7 PFU i.t.) was given on day 7 (when tumors reached approximately $100mm^3$) and the second dose was given on day 10. Treatment groups are: 1. vMyx-IL15Rα-tdTr, 2. vMyx-tdTr, 3. PBS. Mice were euthanized 3 days after the final virus treatment and tumor sections were analyzed for presence of T cells by immunostaining for CD4 and CD8 markers. Representative tumor sections are shown. (A) Staining for $CD8^+$ T cells in tumors. Green – CD8-positive cells, Blue – DAPI. Scale bar = 50 micrometers. (B) Staining for $CD4^+$ T cells in tumors. Red – CD4-positive cells, Blue – DAPI. Scale bar = 50 micrometers.

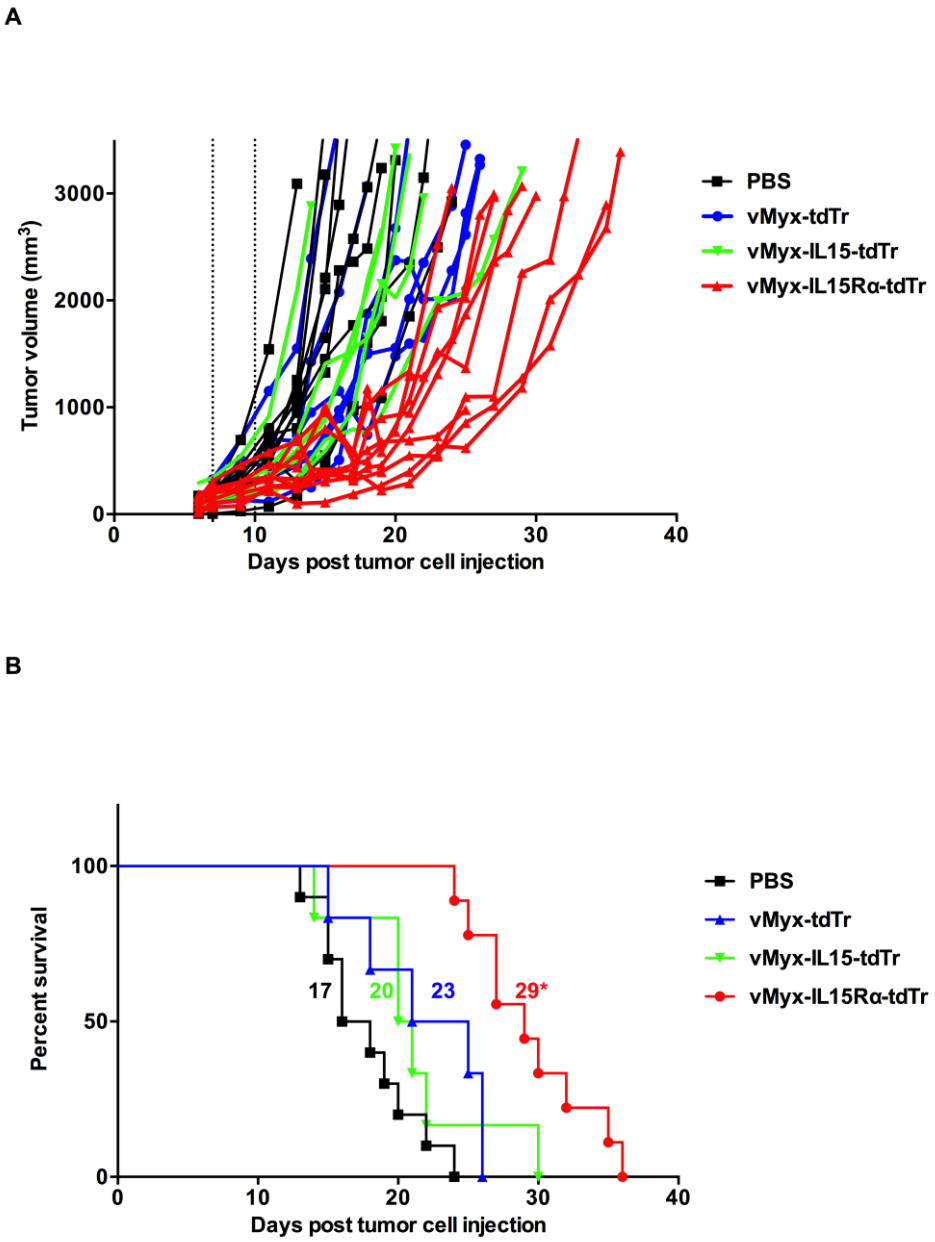

Figure 2.8. Prolonged survival of RAG1$^{-/-}$ mice bearing subcutaneous B16-F10 tumors treated with vMyx-IL15Rα-tdTr intratumorally. RAG1$^{-/-}$ mice (10 mice in vMyx-IL15Rα-tdTr and PBS and 6 mice in vMyx-IL15-tdTr and vMyx-tdTr treatment groups) were implanted with subcutaneous B16-F10 tumor cells. 7 days later, when tumors reached approximately 100 mm^3, virus was inoculated intratumorally (i.t.) with 2.6×10^7 PFU vMyx-IL15Rα-tdTr, vMyx-IL15-tdTr, vMyx-tdTr or PBS. Mice received a second i.t. inoculation of 2.6×10^7 PFU of each virus on day 10. (A) Growth of individual tumors. Dashed lines designate time of virus treatment, and growth of tumors was measured every 2 days. (B) Kaplan–Meier survival curve of the same experimental subjects. Numbers next to corresponding survival curves designate median survival time (days). (* - $p < 0.05$ for vMyx-IL15Rα-tdTr treated group compared to other vMyx, as well as PBS treatment)

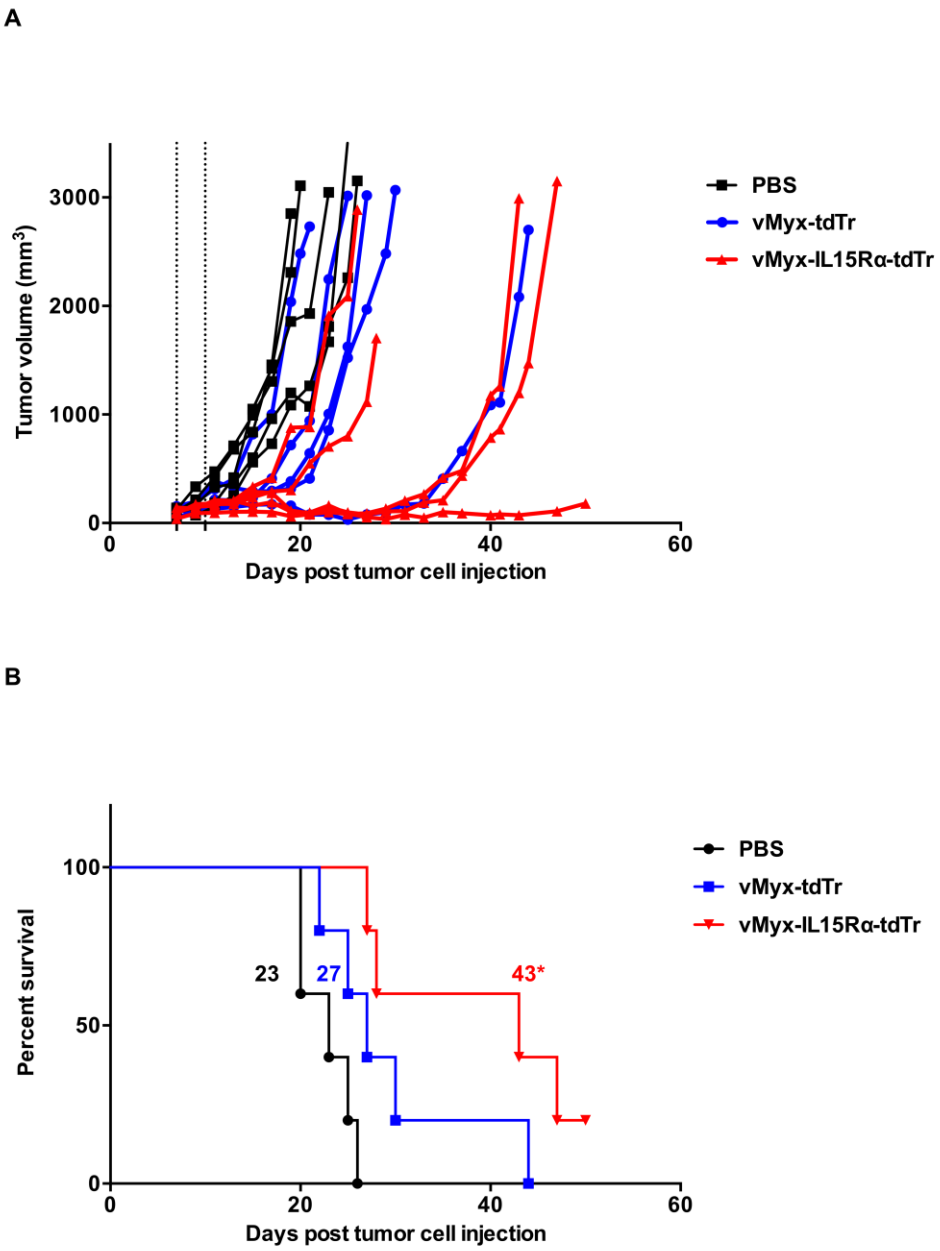

Figure 2.9. Prolonged survival of C57BL/6 mice bearing subcutaneous B16-F10 tumors treated with vMyx-IL15Rα-tdTr intratumorally. C57BL/6 mice (5 mice per group) were implanted with subcutaneous B16-F10 tumor cells. 7 days later, when tumors reached approximately 100 mm^3, virus was inoculated intratumorally (i.t.) with 2.6×10^7 PFU vMyx-IL15Rα-tdTr, vMyx-tdTr or PBS. Mice received a second i.t. inoculation of 2.6×10^7 PFU of each virus on day 10. (A) Growth of individual tumors. Dashed lines designate time of virus treatment, and tumor size was measured every 2 days. (B) Kaplan–Meier survival curve of the same experimental subjects. Numbers next to corresponding survival curves designate median survival time (days). (* - $p < 0.05$ for vMyx-IL15Rα-tdTr treated group compared to PBS)

CHAPTER THREE

MYXOMA VIRUS AND T CELL COMBINATION THERAPY

3.1 Introduction

Adoptive T cell therapy and other immunotherapies for cancer have shown promise as monotherapies, but cancer recurrence remains one of the big issues [1]. Antigen loss variants (ALVs), sub-populations of cancer cells that no longer express the target antigen that may escape destruction by the T cells and cause reemergence of the tumor, remain a challenge for tumor-antigen specific therapies [2]. It is evident that successful treatment of different cancers will have to involve some form of combination therapy [3] [4], and many clinical trials are underway to test combinations of previously approved and new treatments [5] [6] [7].

Oncolytic viruses mostly induce immunogenic cancer cell death, leading to release of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) along with tumor-associated antigens (TAAs) at the tumor site [8] [9]. These events activate dendritic cells and elicit adaptive antitumor immunity [10]. Oncolytic viral therapy also has the potential to increase pro-inflammatory cytokines and chemokines and decrease immunosuppressive cytokines in the tumor environment [11].

Successful elimination of solid tumors and prevention of their recurrence from outgrowth of ALVs is dependent on destroying cancer cells as well as non-transformed stromal cells in tumor environment that support tumor growth [12] [13]. Antigen release from dying cancer cells by irradiation, chemotherapy or other methods can increase presentation of tumor antigen on stromal cells, which contributes to enhanced killing by adoptively transferred T cells [14]. Successful elimination of tumors that expressed high levels of TAAs by cytotoxic T cells was observed in both subcutaneous and brain tumor mouse models [14] [15].

In this study, we first describe results of an experiment in which virus expressing IL15Rα-IL15 construct was combined with naïve $CD8^+$ T cells to treat a murine melanoma, B16-F10. This combination did not produce a pronounced antitumor effect, so we next tested virus therapy in combination with T cells specific for an antigen expressed on the surface of the cancer cells. In order to do that, we used a well characterized 2C T cell system that recognizes SIY antigenic peptide on the context of K^b (mouse MHC molecule) on B16-F10 melanoma cells transduced to express SIY (B16.SIY). The combined therapies showed a significant survival benefit, but mice still succumbed to the tumors, presumably because of antigen loss variants (ALV), as previously described [16] [17]. With the idea of increasing tumor antigen presentation in initial phase of T cell antitumor response, we generated a construct to create a new virus, vMyx-SIY-tdTr, which would have desirable properties of specifically targeting cancer cells, as well as expressing the

SIY antigen in the infected cell and making antigen available to the tumor stroma. We hypothesized that combination of viral effect of enhancing the attenuated immune response and increasing presence of T cell specific antigen in the tumor would lead to better control of the tumor, or even tumor-free survival. Here we describe initial phases of the creation of the new virus.

3.2 Materials and methods

DNA construct

pBS-IL15Rα-IL15-tdTomatoRed, used for generating vMyx-IL15Rα-tdTr was described in the previous chapter. Plasmid for expressing SIY and tdTomato as a single transcriptional unit under the vvSynE/L promoter, termed pBS-SIY-tdTomato, was created in a similar fashion. Briefly, PCR product isolated from vMyx-tdTr [18] was ligated into pBluescript (ATCC, Manassas, VA) using the T4 Ligase (Invitrogen, Carlsbad, CA) following sequential digestion with HindIII and BamHI (New England BioLabs, Ipswich, MA). tdTomato was cloned out of the plasmid provided by Dr. Brian Freeman (University of Illinois) using primers: forward 5'- GCA AGA TCT ATG GTG AGC AAG G – 3' and reverse: 5'- CCT GAA TTC TTA CTT GTA CAG CTC G – 3' and cloned into the existing pBluescript construct using BglII and EcoRI (New England BioLabs, Ipswich, MA). SIYRYYGL triplicate sequence was isolated from pMFG-SIY-EGFP (similar to [12]) using primers: forward 5'- CGG GGG TGG ACC ATC CTC TAG AGA TCT ATG TTG – 3' and reverse: 5'- GCA AGA TCT GGT GGC GAC CGG TGG ATC – 3' and cloned into the existing construct using a single BglII restriction site. Orientation and copy number of SIY triplicates was confirmed by sequencing the resulting pBS-SIY-tdTomato plasmid (Figure 3.4). This construct will eventually be used to engineer the SIY-tdTomato expressing myxoma virus.

Recombinant virus

Creation and characterization of recombinant vMyx-IL15Rα-tdTr was described in detail in the previous chapter. Briefly, vMyx-IL15Rα-tdTr was created by homologous recombination in RK-13 cells infected with wild type (WT) vMyx-Lau followed by transfection with the engineered recombination vector pBS-IL15Rα-IL15-tdTomatoRed. Genes for the IL15Rα-IL15 fusion protein and tdTomato, both under control of identical vvSynE/L promoters, were inserted between M135 and M136 myxoma virus genes.

Cell culture and reagents

The murine melanoma cell line, B16-F10 was purchased from ATCC (Manassas, VA). B16.SIY was derived from B16-F10 cells retrovirally transduced to express green fluorescent protein (GFP) as a fusion protein with SIYRYYGL (SIY) [12,19] and was a gift from Dr. Thomas Gajewski (University of Chicago, Chicago, IL).

Cancer cell lines were cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium containing 5 mM HEPES, 1.3 mM L-glutamine, 50 μM 2-ME, penicillin, streptomycin and 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Animals

C57BL/6 and C57BL/6 RAG1$^{-/-}$ mice originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were maintained as colonies and housed in the animal facilities at the University of Illinois. Colonies of 2C T cell receptor (TCR) transgenic mice on the C57BL/6 background (provided by J Chen, Massachusetts Institute of Technology, Cambridge, MA) are maintained as a heterozygous colony and screened for expression of the 2C TCR on Thy1.2^{+} peripheral blood cells with 1B2 clonotypic antibody by flow cytometry. Mice were used in experiments when they were 2-5 months old. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign (PHS Assurance A3118-01, AAALAC, International Accreditation #00766). Anesthesia was used during tumor cell and virus injections, and all efforts were made to minimize suffering.

Subcutaneous tumor establishment and treatment

B16-F10 melanoma cells were harvested and washed twice with Hanks Balanced Salt Solution (HBSS, Cellgro Mediatech Inc., Manassas, VA). Prior to all procedures, mice were anesthetized by isoflurane (Aerrane, Baxter, Deerfield, IL) inhalation using the classic vaporizer unit by E-Z Systems (Palmer, PA). Shaved mice received 1x10^{6} tumor cells in 100µl HBSS subcutaneously into the right flank. After 7 days, when tumors usually reach a volume of approximately 100 mm^{3}, mice were assigned to treatment groups and received an intratumoral (i.t.) injection of virus. At this time, tumors were directly injected with 2.6×10^{7} PFU of sucrose-pad purified vMyx-IL15α-tdTr or PBS that was in a final volume of 50 µl. An additional i.t. inoculation of the virus (2.6×10^{7} PFU) or PBS occurred 3 days later (day 10 post-implantation). For those tumors that were large, the inoculum was injected into at least three different sites to introduce the virus throughout the mass. Prior to all repeated tumor injections with virus or PBS, animals were anesthetized by isoflurane inhalation as described earlier. All animals were single housed upon tumor cell implantation and during all subsequent experimental manipulations.

Tumor growth was monitored by measuring tumor length, width and height with a caliper. Tumor volume was calculated as ((length) x (width) x (height))/2. Mice were monitored daily. Mice were humanely euthanized when tumors reached the volume of 3000mm^{3}, or showed lethargy or signs of pain, or when reaching 75% baseline body weight. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation.

Intracranial tumors establishment and treatment

B16-SIY melanoma cells were harvested, washed twice with HBSS and stereotaxically infused into the brains of mice anesthetized with isoflurane, as described above. 2x10^{3} B16.SIY cells in 300 nL HBSS were

infused into ventral striatum (from bregma: 0.5 mm rostral; 2.5 mm lateral; 4 mm ventral). Mice were euthanized at 75% of baseline body weight or signs of neurological impairment, lethargy or pain, in accordance with IACUC guidelines.

Tumor bearing mice were stereotaxically injected intratumorally (i.t.) with approximately $1x10^6$ PFU vMyx-IL15α-tdTr or PBS control in 0.7µL PBS. Some mice were euthanized 4 days post virus injection for analysis of tdTomatoRed expression (this time point was based on our previous studies [16]).

For adoptive 2C T-cell transfer, lymphocytes from spleens and lymph nodes of 2C TCR mice were prepared by mechanical tissue dissociation through nylon mesh followed by ACK buffer lysis of erythrocytes. Mixed lymphocytes were incubated at 37°C, 5% CO_2 for 48h with 1µM SIY peptide and 5% rat ConA supe (RCAS) to activate and expand effector 2C T cells. Cells were then collected, washed with HBSS and an average of $5x10^6$ of activated 2C T cells in 200µl HBSS was injected into the tail vein of mice 6 days following tumor cell infusion. Control mice were injected with 200µL HBSS.

Tissue sections and immunostaining

After the mice were euthanized, their brains with intracerebral tumors and draining lymph nodes were immersion fixed in 10% formalin to preserve tdTomato protein expression, and then snap-frozen in OCT medium for cryosectioning and immunostaining. Eight µm cryosections were taken. For tdTomatoRed visualization, slides were washed in filtered dH_20 and PBS and briefly incubated with DAPI. Images were obtained with an Olympus BX-51 microscope at 10x and 20x magnification.

Transient transfection and MVA infection

B16-F10 and B16.SIY cells were plated in 6-well culture plates and upon reaching 90-95% confluency they were transfected with pBS-SIY-tdTomato or pBS-IL15Rα-IL15-tdTomato using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 9h after transfection with plasmid DNA, cells were infected with modified vaccinia Ankara (MVA) poxvirus (gift from Dr. Joanna Shisler's laboratory, University of Illinois) at MOI=5. Inoculated cells were incubated with the virus in serum-free media at 37°C and 5% CO_2 for 1h, rocking every 15 min. Cells were imaged and collected at 12 h and 36 h post-infection (p.i.). Fluorescent images in the Texas Red channel were taken by Olympus DP12 camera and cells were collected and analyzed by flow cytometry for presence of transiently expressed molecules.

Flow cytometry

Plasmid DNA transfected and MVA infected B16-F10 and B16.SIY cells in 6-well plates were trypsinized, washed in PBS and prepared for analysis on the Accuri C6 instrument. tdTomato expression was detected

in the FL2 channel. Additionally, cells were stained for the expression of mouse MHC Class I molecule K^b (anti-K^b-APC, eBioscience, San Diego, CA) and for the K^b-SIY complex using biotinylated soluble high-affinity TCR, m67 [14] (provided by Dr. Jennifer Stone, Kranz laboratory, University of Illinois), followed by Streptavidin-APC (Invitrogen, Carlsbad, CA). Data were analyzed on the FCS Express software.

Statistics

GraphPad Prism software (La Jolla, CA) was used for all statistical analyses and graph presentation. Survival data were recorded from the time of the tumor cell implantation until euthanasia and were plotted using a Kaplan-Meier curve. Survival treatment groups were compared with a Log-rank (Mantel-Cox) test. Significance was considered $p < 0.05$.

3.3 Results

Treatment with IL15Rα-IL15 fusion protein expressing myxoma virus and naïve T cells provides a survival benefit to mice with subcutaneous tumors

In vitro assays described in previous chapter showed that vMyx-IL15Rα-tdTr is able to productively infect cells in culture and render them capable of secreting IL15Rα-IL15 fusion protein. We also showed an effect of the virus as a stand-alone therapy in $RAG1^{-/-}$ and C57BL/6 mice. The next step was to test the virus *in vivo* as a combination therapy with adoptively transferred immune cells. $RAG1^{-/-}$ mice bearing established B16-F10 tumors were treated with vMyx-IL15Rα-tdTr intratumorally alone or in combination with primary murine naïve cytotoxic T cells ($CD8^{+}$ cells) intravenously. Treatment with cytokine fusion protein expressing vMyx-IL15Rα-tdTr significantly contributed to survival of mice bearing B16-F10 subcutaneous tumors, similarly as shown in the previous chapter (median survival of 36 days compared to 21 days for PBS only treated mice, Figure 3.1). Naïve cytotoxic T cells did not significantly contribute to increased survival of tumor bearing animals, either alone or in combination with the virus, although the two longest surviving mice were treated with the combination.

Treatment with vMyx-IL15Rα-tdTr in combination with 2C T cells provides a survival benefit to mice with intracranial tumors

After encouraging viral therapeutic effect with subcutaneous tumors, we sought to investigate the potential therapeutic effect of vMyx-IL15Rα-tdTr on established brain tumors. We also investigated a possible synergistic effect between the immunostimulatory protein expressing virus and tumor-specific T cells. For this purpose, we used the well-established 2C system, in which cytotoxic T cells equipped with the 2C T cell receptor specifically recognize and kill cancer cells engineered to express the SIY peptide [20]. Figure 3.2 shows the survival of mice bearing B16.SIY intracranial tumors after treatment with vMyx-IL15Rα-tdTr intratumorally and/or 2C T cells intravenously. We know from previously published studies [16] that 2C T cell clone by itself prolongs survival of tumor bearing mice beyond 30 days after tumor cell implant in this model. Addition of the virus treatment showed a trend toward increased survival compared to the 2C T cell only treated group (median survival of 37.5 vs. 33.5 days, respectively). All treated groups survived significantly longer than the PBS control.

Figure 3.3 shows productive and selective infection of brain tumor tissue 4 days after virus treatment. Virally encoded fluorescent protein, tdTomato is expressed in virus infected tumor cells. No notable difference in virus spread was observed in the treatment group that was treated with 2C T cells in addition to the virus.

tdTomato is expressed from the pBS-SIY-tdTomato plasmid in transiently transfected cells

With the idea of boosting the antitumor immunity by increasing the antigen presentation in the model tumor antigen SIY – 2C T cell system, we decided to create a myxoma virus that would specifically express SIY in the context of the tumor. After successfully cloning the fusion protein of SIY repeats and tdTomato into the pBS plasmid, the new construct, pBS-SIY-tdTomato was tested to ensure expression of the desired proteins from the virus-infected cells. In order to do that, we did a transient transfection of B16-F10 and B16.SIY cells with the plasmid, coupled with the infection by the MVA virus. Infection by a poxvirus MVA was necessary in order to drive expression of the genes from the viral promoter.

tdTomato was visible by fluorescent microscopy in the Texas Red channel in transiently transfected and virus infected cells at 12 h p.i. (Figure 3.5A), and in even more cells at 36 h p.i. (Figure 3.5B). The trend of tdTomato expression in treated B16-F10 cells was confirmed by flow cytometry. At 12 h p.i., about 16% of cells were estimated positive for tdTomato (Figure 3.6A), while the fraction of tdTomato expressing B16-F10 cells was close to 33% at 36 h p.i. (Figure 3.6B). Cells transfected with pBS-IL15Rα-IL15-tdTomato served as a positive control for tdTomato expression.

B16.SIY cells were previously engineered to express SIY peptide [12], and were intended as a positive control for K^b-SIY complex staining by the high affinity TCR m67 [14]. Untreated cultured B16-F10 and B16.SIY cells express an undetectable level of the relevant MHC molecule, K^b (Figures 3.7A and B, black histograms). Transfection with the DNA and MVA infection led to upregulation of K^b on the surface of the cells; however DNA transfection alone caused the highest expression of K^b above background (75% positive cells), while simultaneous MVA infection somewhat reduced MHC expression (45% positive cells)(Figure 3.7A). Similar results were observed in B16.SIY cells (Figure 3.7B). Staining for K^b-SIY with soluble m67 TCR suggested surface presentation of the SIY peptide (7% of pBS-SIY-tdTomato and MVA treated B16-F10 cells were positive, compared to less than 1% for untreated B16-F10 cells, Figure 3.7C). Lack of K^b-SIY staining on constitutively expressing B16.SIY cells (Figure 3.7D, black histogram) could be due to low MHC levels in the no treatment group (compare with figure 3.7B). However, K^b-SIY should have been detectable in B16.SIY cells that had increased level of K^b (Figure 3.7D, green histogram). Modified treatment conditions and pretreatment with IFNγ will be necessary to unequivocally confirm K^b-SIY staining on the surface of the cells.

3.4 Discussion

It has been previously shown in our laboratory and by others that increasing tumor antigen expression can lead to tumor eradication. In the brain tumor model, adoptively transferred cytotoxic 2C T cells specific for the SIY peptide antigen presented by the cancer cells completely eliminated SIY-high expressing brain tumors and cured mice while mice with SIY-low tumors usually relapsed [15]. Tumor-antigen specific T cells were capable of eliminating even allogeneic tumors expressing the same antigen but non-cognate MHC molecules (H-2^k), which prevents 2C T cells from directly recognizing them. This result confirmed that cross-presentation of the tumor antigen by stromal cells played a significant role in tumor elimination by the adoptively transferred T cells. Similarly, in the subcutaneous model, if cancer cells express only low levels of SIY, stromal cells are not destroyed by the transferred 2C T cells, and the tumor escapes as ALVs [14]. In addition, this study showed that treating tumors expressing low levels of antigen with local irradiation or a chemotherapeutic drug caused sufficient release of the antigen to sensitize stromal cells for destruction by T cells and subsequent tumor rejection. Proper timing of the T cell transfer was essential for the combination therapy success, since loading and cross-presentation of tumor antigen by the stromal cells *in vivo* peaked at 2 days after radiation or chemotherapy. We postulate that oncloytic effect of the virus could have a similar effect as radiation or chemotherapy observed in this study. The SIY-expressing construct needs to be inserted into the myxoma virus to allow testing of this hypothesis.

The feasibility of combining an oncolytic virus with adoptive T cell therapy has been demonstrated by many groups [9] [21]. The Vile group showed that vesicular stomatitis virus (VSV) engineered to express chicken ovalbumin (OVA) mediated complete regression of B16 melanomas expressing OVA antigen in many mice, since treatment with recombinant virus developed potent anti-OVA immunity [22]. In another study by the same group, ACT combined with systemic administration of two engineered VSVs (OVA and endogenous melanocyte antigen glycoprotein 100 (gp100)-expressing variants) induced regression of established tumors in all treated mice [23]. Using a different strategy, chemokine CCL5-expressing vaccinia virus in combination with a cancer vaccine or activated T cells improved therapeutic effect in a murine colon cancer model [24]. Another group used an oncolytic vaccinia virus engineered to express a secretory bispecific T cell engager, an antibody based construct that recognizes the tumor cell surface antigen EphA2 and CD3 molecule on the surface of T cells. The molecule secreted by the virus activates T cells in the proximity of the tumor, and it showed potent antitumor activity in the lung cancer xenograft model [25]. A study from our laboratory showed promise in combining adoptive immunotherapy and rapamycin-enhanced virotherapy, since rapamycin treatment did not impair T cell-mediated tumor destruction [16].

Virus might act as an effective adjuvant for the expression of TAA to adoptively transferred, TAA-specific T cells by activation of antigen presenting cells (APCs) through signaling molecules such as MyD88, as

well as Type I and Type III interferon mediated signaling [23]. $CD8\alpha^+$ dendritic cells are particularly effective at acquiring antigen from dying cells, including tumor cells, and targeting antigen for cross-presentation via the class I MHC processing pathway [26].

Therapeutic approaches exploring multiple ways of activating antitumor immune response and enhancing antigen presentation are under active development. A poxvirus based therapeutic cancer vaccine that is currently in the phase III clinical trials, PROSTVAC , is designed to break immunological tolerance to prostate specific antigen (PSA) and initiate a robust immune response against prostate cancer [27]. Vaccinia and fowlpox virus based vectors are engineered to express PSA and a triad of human T-cell costimulatory molecules (the TRICOM triad: B7.1, ICAM-1, and LFA-3) [28]. Preclinical studies have shown that the vaccine's ability to treat tumors is largely dependent on $CD8^+$ T cells, and partially dependent on $CD4^+$ T cells and natural killer (NK) cells [29]. Current clinical studies are analyzing the vaccine's ability to generate tumor-specific T cells.

Additionally, a combination of oncolytic viruses with immunotherapeutic strategies aimed to disrupt tumor-induced tolerance is an attractive strategy. In one recent study, the combination of replication competent VSV with anti-CTLA-4 monoclonal antibody led to elimination of tumor implants in the majority of animals, in a $CD4^+$ and $CD8^+$ T cell dependent manner [30]. In another very recently published study, localized therapy with oncolytic Newcastle disease virus (NDV) induced inflammatory immune infiltrates in distant tumors, making them susceptible to systemic therapy with an anti-CTLA-4 antibody. The effect was dependent on $CD8^+$ cells, NK cells and type I interferon [31]. These studies provide strong evidence that such combinations would be useful in the clinical setting.

In summary, an ideal immunotherapeutic intervention against cancer would have to (1) stimulate the presentation of TAAs to T cells, while (2) counteracting the immunosuppressive activity of tumor microenvironment [32]. We believe that combination therapy of TAA-expressing virus and ACT may have synergistic effects and will lead to improved effectiveness of both treatments.

3.5 References

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3.6 Figures

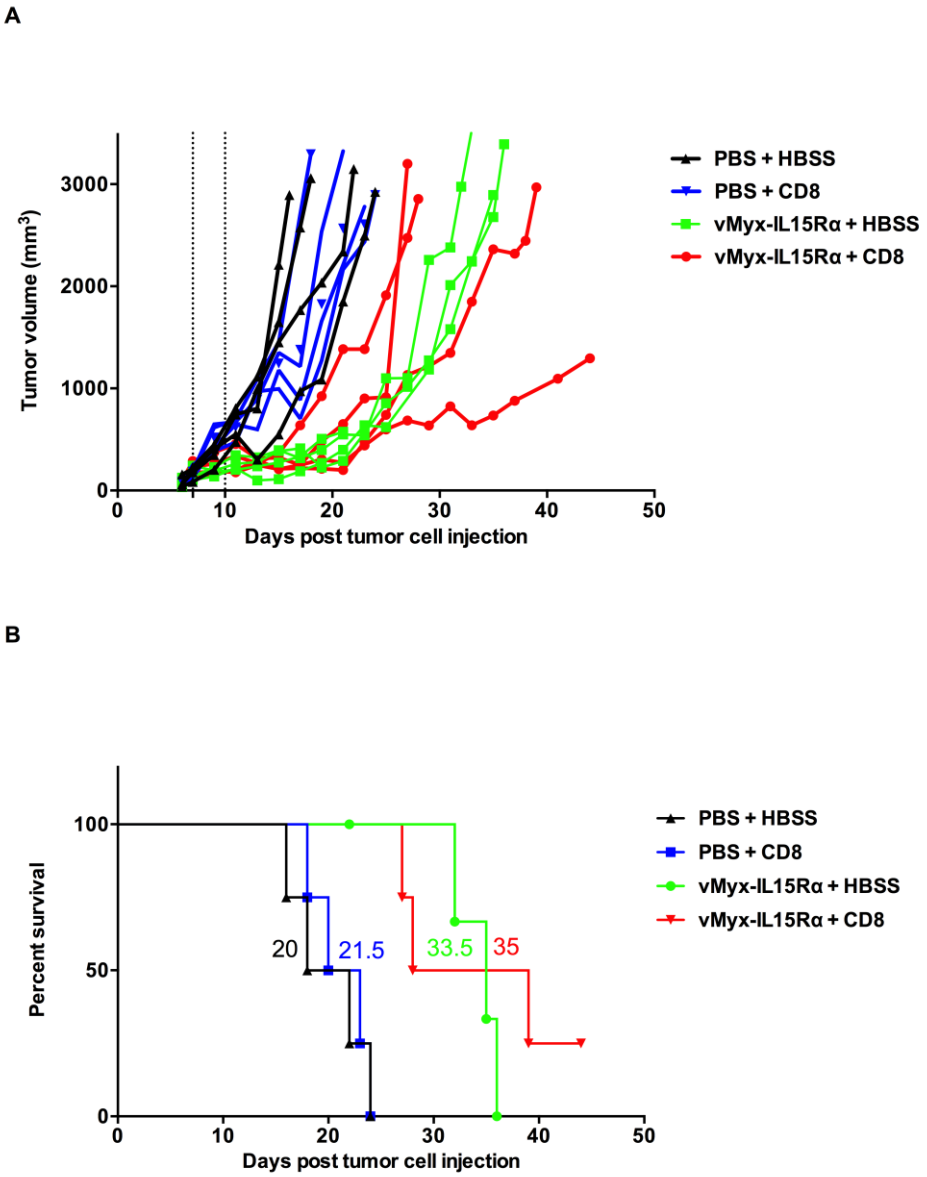


Figure 3.1 – Survival and tumor growth in RAG1$^{-/-}$ mice with subcutaneous B16-F10 treated with vMyx-IL15Rα-tdTr and naïve CD8^{+} T cells. RAG1$^{-/-}$ mice were implanted with unilateral subcutaneous B16-F10 tumor cells. Adoptively transferred naïve CD8^{+} T cells i.v. and the first dose of the virus (2.6×10^{7} PFU i.t.) were given on day 7 (when tumors reached approximately 100mm^{3}). Second dose of the same amount of virus only was given on day 10. Treatment groups are: 1. vMyx-IL15Rα-tdTr i.t. + CD8^{+} T cells i.v., 2. vMyx-IL15Rα-tdTr i.t. + HBSS i.v., 3. PBS i.t. + CD8^{+} Tcells i.v., 4. PBS i.t. + HBSS i.v. Total number of mice were 4 per group, with the exception of n=3 for vMyx-IL15Rα-tdTr only treatment group. (A) Growth of individual tumors. Dashed lines designate time of virus treatment (B) Kaplan–Meier survival curve of the same experimental subjects.

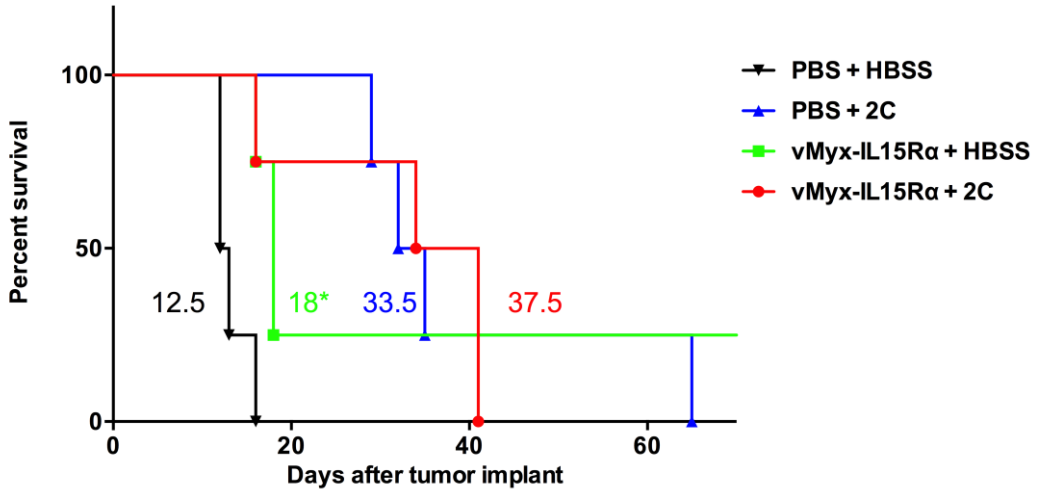


Figure 3.2 – Survival of RAG1$^{-/-}$ mice with intracranial B16-SIY treated with vMyx-IL15Rα-tdTr and 2C T cells. RAG1$^{-/-}$ mice were implanted with unilateral B16-F10 tumor cells into the right ventral striatum. On day 5, mice were treated with a single dose of 1x10^{6} PFU vMyx-IL15Rα-tdTr i.t. On day 6, 5x10^{6} activated 2C T cells were adoptively transferred i.v. to the tumor-bearing mice. Treatment groups are: 1. vMyx-IL15Rα-tdTr i.t. + 2C T cells i.v., 2. vMyx-IL15Rα-tdTr i.t. + HBSS i.v., 3. PBS i.t. + 2C Tcells i.v., 4. PBS i.t. + HBSS i.v. Kaplan–Meier survival curve; N=4 per group (* - $p<0.05$ for vMyx-IL15Rα-tdTr/HBSS group compared to the PBS/HBSS treated group)

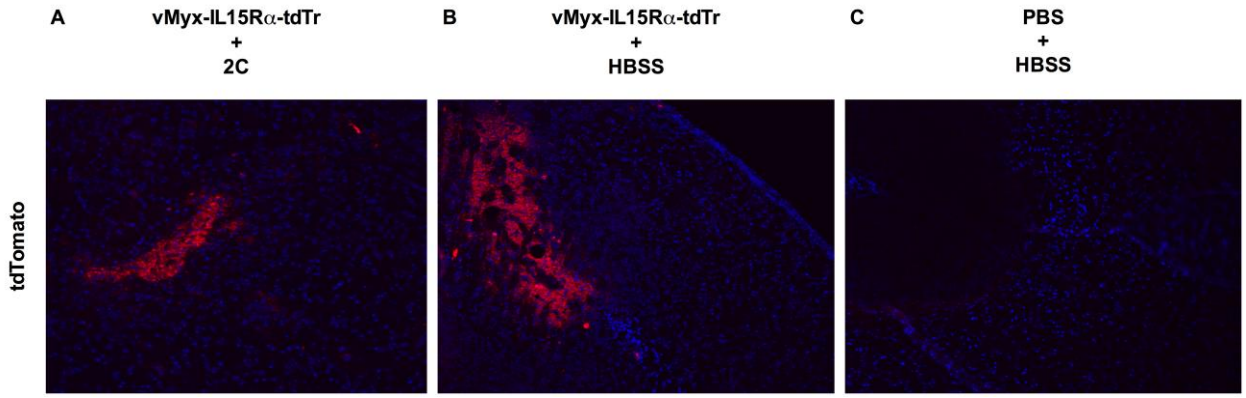


Figure 3.3 – tdTomato expression in the B16-SIY brain tumor sections of RAG1$^{-/-}$ mice 7 days after treatment with vMyx-IL15Rα-tdTr intratumorally. RAG1$^{-/-}$ mice were implanted with B16-SIY tumor cells unilaterally into the right ventral striatum. Five days later mice were treated by a single dose of vMyx-IL15Rα-tdTr i.t. On day 5, mice were treated with a single dose of 1x10^6 PFU vMyx-IL15Rα-tdTr i.t. On day 6, 5x10^6 activated 2C T cells were adoptively transferred i.v. to the tumor-bearing mice. On day 12, mice were euthanized; brains were fixed, frozen in embedding media, and cryosectioned. Representative brain tumor sections are shown: (A) vMyx-IL15Rα-tdTr i.t. + 2C T cells; (B) vMyx-IL15Rα-tdTr i.t. + HBSS i.v.; (C) PBS i.t. + HBSS i.v. Red – tdTomato fluorescent protein; Blue – DAPI.

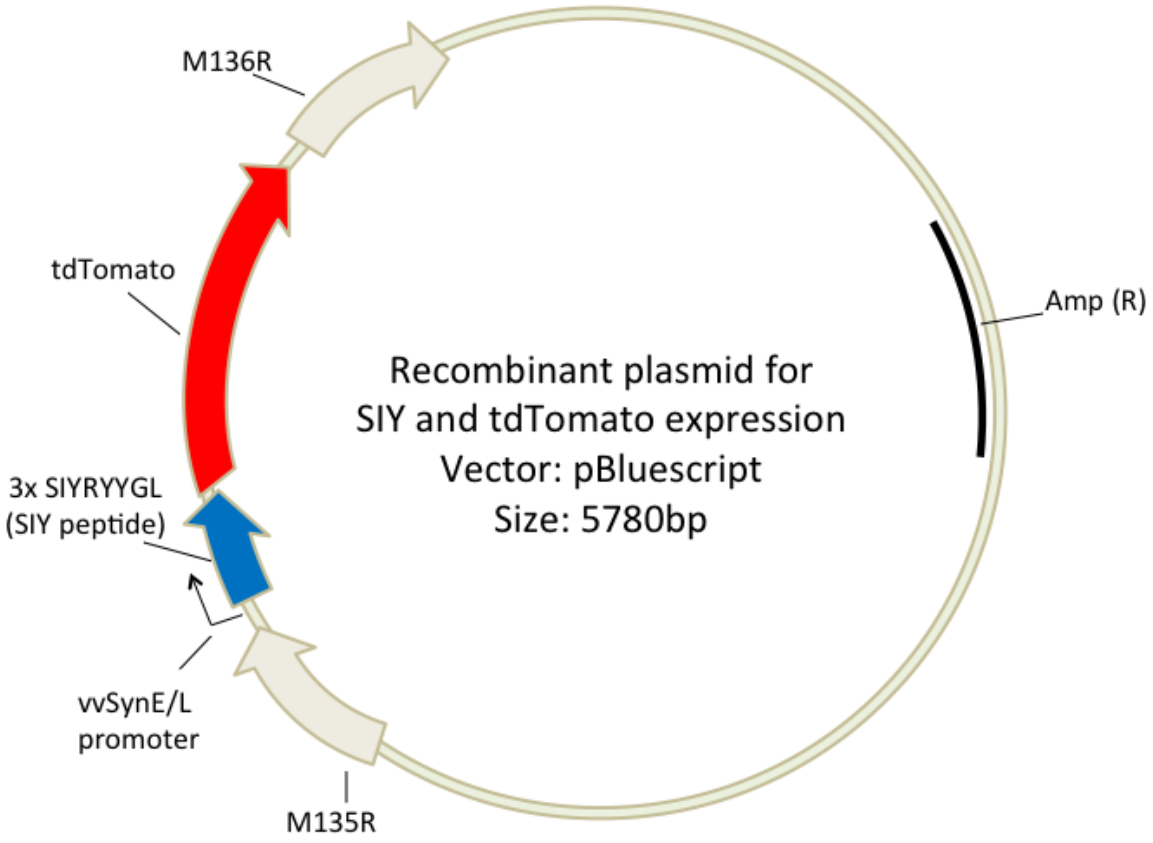


Figure 3.4 – Recombinant plasmid for modifying WT myxoma virus and generating vMyx-SIY-tdTr (pBS-SIY-tdTomato). Plasmid pBS-SIY-tdTomato (5780bp) is based on the pBluescript backbone on which M135 and M136 partial viral gene sequences are flanking genes for $(SIYRYYGL)_3$-9 amino acid linker- tdTomato fluorescent protein transcription unit, under control of the vvSynE/L poxviral promoter. The expression cassette is flanked by partial viral gene sequences for the purpose of being transfected into the WT vMyx-Lau virus genome between genes M135 and M136.

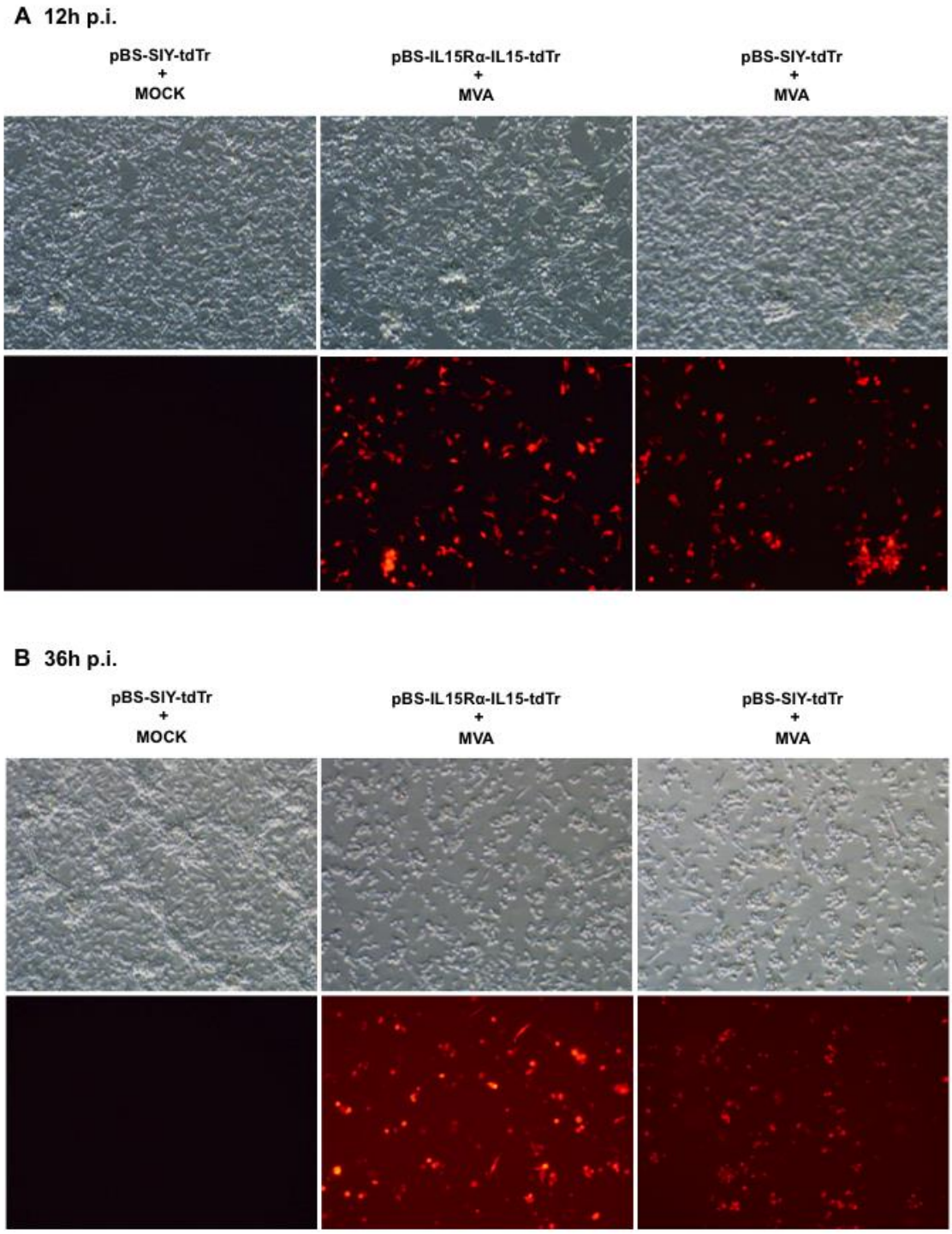
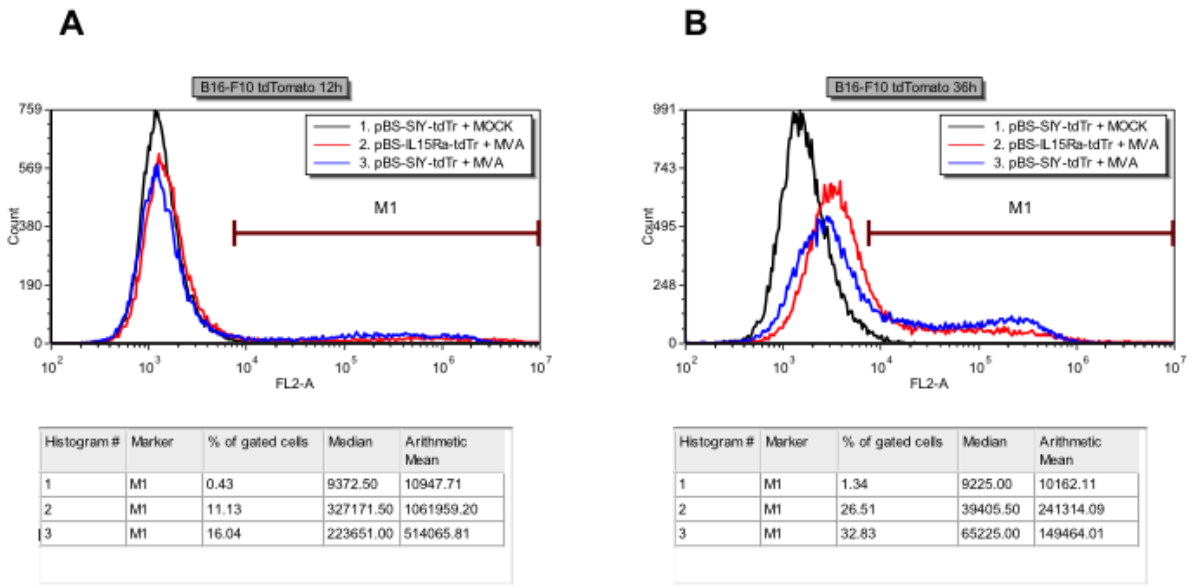


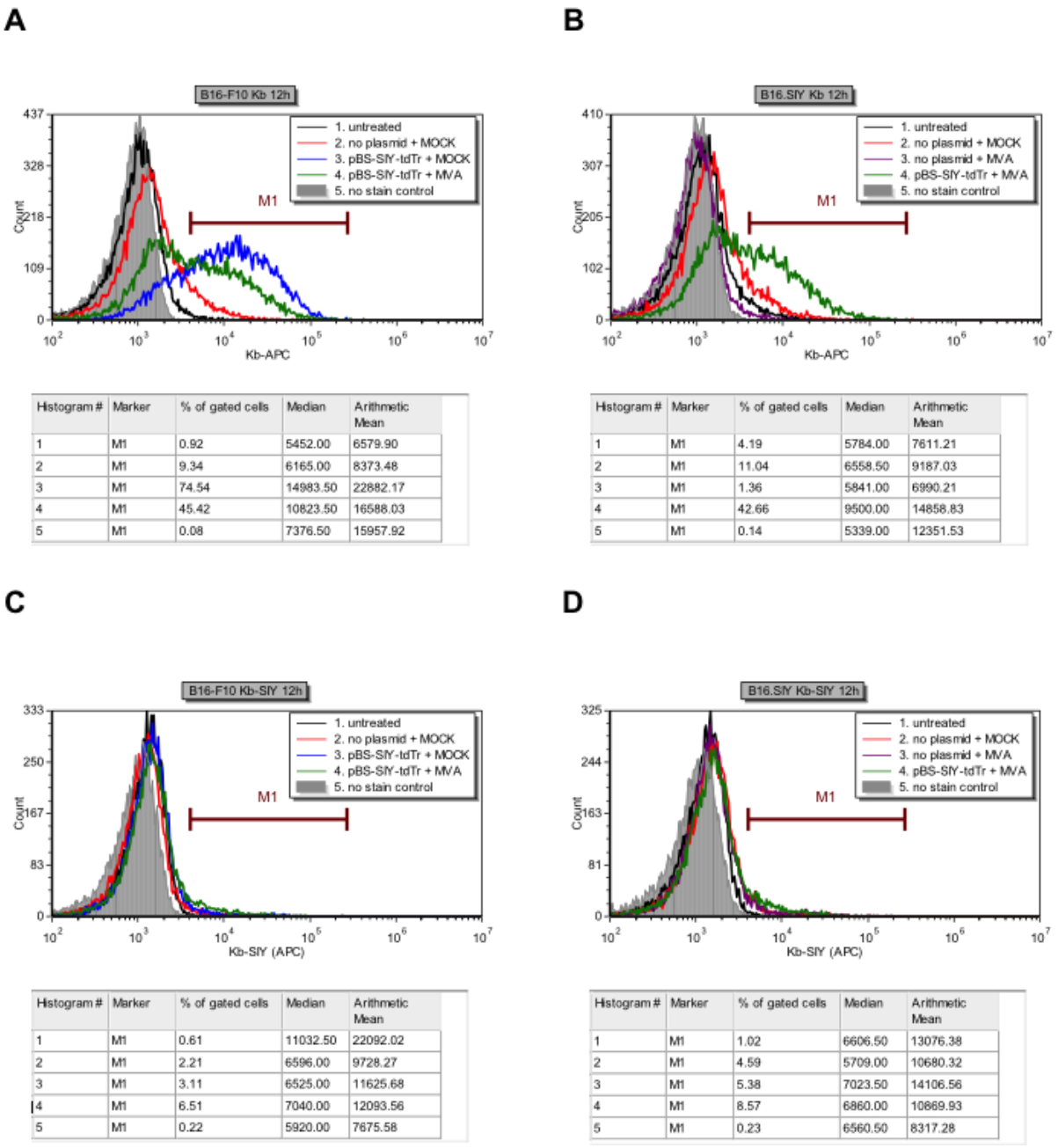
Figure 3.5 – B16-F10 cells transiently transfected with pBS-SIY-tdTomato and infected with a poxvirus (MVA) express tdTomato fluorescent protein. B16-F10 cells in 6-well culture plates were transfected with plasmid DNA; 9h later, cells were infected with MVA at MOI=5. Treatment groups (Plasmid + Virus) are: 1. pBS-SIY-tdTomato + mock; 2. pBS-IL15Rα-IL15-tdTomato + MVA; 3. pBS-SIY-tdTomato + MVA. Cells were imaged at (A) 12 h p.i. and (B) 36 h p.i. Top and bottom panels present bright-field and fluorescent image, respectively. Red fluorescence– tdTomato



Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	0.43	9372.50	10947.71
2	M1	11.13	327171.50	1061959.20
3	M1	16.04	223651.00	514065.81

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	1.34	9225.00	10162.11
2	M1	26.51	39405.50	241314.09
3	M1	32.83	65225.00	149464.01

Figure 3.6 – Flow cytometry detection of tdTomato expressed by B16-F10 cells transiently transfected with pBS-SIY-tdTomato and infected with MVA. B16-F10 cells in 6-well culture plates were transfected with plasmid DNA; 9h later, cells were infected with MVA at MOI=5. Treatment groups (Plasmid + Virus) are: 1. pBS-SIY-tdTomato + mock; 2. pBS-SIY-tdTomato + MVA; 3. pBS-IL15Rα-IL15-tdTomato + MVA. Cells were trypsinized, washed and analyzed for tdTomato expression in the FL2 channel. (A) 12 h p.i. (B) 36 h p.i. Percentages in all histogram legends indicate fraction of gated cells in the designated M1 marker.



Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	0.92	5452.00	6579.90
2	M1	9.34	6165.00	8373.48
3	M1	74.54	14983.50	22882.17
4	M1	45.42	10823.50	16588.03
5	M1	0.08	7376.50	15957.92

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	4.19	5784.00	7611.21
2	M1	11.04	6558.50	9187.03
3	M1	1.36	5841.00	6990.21
4	M1	42.66	9500.00	14858.83
5	M1	0.14	5339.00	12351.53

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	0.61	11032.50	22092.02
2	M1	2.21	6596.00	9728.27
3	M1	3.11	6525.00	11625.68
4	M1	6.51	7040.00	12093.56
5	M1	0.22	5920.00	7675.58

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	1.02	6606.50	13076.38
2	M1	4.59	5709.00	10680.32
3	M1	5.38	7023.50	14106.56
4	M1	8.57	6860.00	10869.93
5	M1	0.23	6560.50	8317.28

Figure 3.7 – Detection of K^b and K^b-SIY on B16-F10 and B16-SIY cells transiently transfected with pBS-SIY-tdTomato and infected with MVA. B16-F10 cells in 6-well culture plates were transfected with plasmid DNA; 9h later, cells were infected with MVA at MOI=5. Treatment groups (Plasmid + Virus) are: 1. untreated cells; 2. no plasmid + mock 3. pBS-SIY-tdTomato + mock OR no plasmid + MVA; 4. pBS-SIY-tdTomato + MVA . At 12h p.i., cells were trypsinized, washed and stained for the expression of mouse MHC Class I molecule with anti-K^b-APC and for the K^b-SIY complex using biotinylated soluble high-affinity TCR, m67 followed by Streptavidin-APC. (A) K^b on B16-F10 (B) K^b on B16.SIY (C) K^b-SIY on B16-F10 (D) K^b-SIY on B16.SIY. Percentages in all histogram legends indicate fraction of gated cells in the designated M1 marker

CHAPTER FOUR

IMMUNOTHERAPY AND GRAFT-VERSUS-HOST DISEASE (GVHD)

4.1 Introduction

Recent clinical trials with adoptive cell transfer (ACT) immunotherapies for cancer have shown promising results, most notably improving cure rates for metastatic melanoma [1]. Genetic engineering of T cells to express conventional α/β T cell receptors (TCRs) or chimeric antigen receptors (CARs) provides an opportunity to make ACT possible without the need to isolate and expand tumor-reactive T cells from individual patients [2]. In addition, TCRs or CARs can be used to redirect either $CD4^+$ helper T cells or CD8+ cytotoxic T cells (CTLs) [3].

Despite successes with ACT, there are safety concerns associated with gene therapy with genetically engineered T cells. Major safety concerns include: choice of an effective and safe target for immunotherapy; potential toxicities associated with the introduced novel TCR; and choosing an appropriate adjuvant strategy to enhance clinical efficacy of TCR gene therapy of cancer [4]. An ideal target antigen for TCR gene therapy should have a high degree of tumor-specific expression in order to limit the chances of damage to normal tissues [4]. It should also be preferably expressed on tumor-initiating cancer stem cells [5] and it should have an essential role in maintaining the malignant phenotype in order to minimize the risk of tumor escape.

A graft-versus-host reaction is also possible with adoptive T cell therapy. In graft-versus-host reactions, an immune attack is directed by the donor cells against normal host tissue. Clinical presentation in humans is usually associated with hematopoietic stem cell transplantation (SCT) for the treatment of cancers such as leukemia [6]. Acute GVHD (reaction within 100 days of graft transfusion) is generally accompanied by tissue damage of the skin, liver, and the gastrointestinal tract while chronic GVHD (reaction delayed for more than 100 days) targets a wide array of organs and can bear similarities with autoimmune disorders [7]. The current standard of care for GVHD prevention after SCT includes combined administration of tacrolimus and methotrexate; however severe GVHD is a major cause of morbidity and mortality following SCT [8].

GVHD-like symptoms observed in our studies and elsewhere are associated with the $CD4^+$ subpopulation of T lymphocytes. Physiologically, $CD4^+$ T cells recognize peptides presented in the context of MHC class II molecules on the surface of antigen presenting cells (APCs). In animal models of GVHD, CD8+ T cells require interaction with cognate antigen to produce GVHD, but $CD4^+$ T cells do not require cognate antigen [9]. Similarly, transfer of $CD4^+$ T cells of undefined specificity is used as an animal model of colitis [10].

With the advent of genetically engineered T cells, it is possible to redirect $CD4^+$ cells to recognize peptides presented by class I MHC molecules, broadening the scope of adoptive cell therapy. Inclusion of $CD4^+$ cells help with primarily cytotoxic $CD8^+$ cells improves the therapeutic effect of ACT in many models [11] and in the clinic [12] [13]. However this could increase the risk of GVHD. Fine tuning the benefits of $CD4^+$ cell help and undesirable side effects will be a necessity in ACT that aims to combine two T cell populations.

We have encountered GVHD-like symptoms in our laboratory as a side effect of cancer therapy with genetically engineered TCRs introduced into $CD4^+$ T lymphocytes [Soto CM, unpublished data]. Briefly, $RAG1^{-/-}$ mice with melanoma or glioma tumors expressing the SIY antigen treated with 2C and m33 TCR transduced T cells developed treatment related symptoms several weeks after ACT. Symptoms included progressive decreased motility and activity, skin lesions involving the eye, ears and tail, hunched posture, and diarrhea. Immunostaining for $CD3^+$ cells showed an abnormal abundance of T lymphocytes dispersed throughout the ear dermis of mice. Histopathological findings included abnormal leukocyte infiltrates in the lungs, livers, stomach, and the small intestine, strongly indicative of GVHD pathology [7].

The goal of this study was to better understand the mechanisms involved in inducing GVHD, in order to improve the outcome of TCR gene-modified T cell therapy while maintaining effective antitumor responses. In order to dissect the cause of the symptoms in mice that received genetically modified T cells, we were interested in testing the following hypotheses: 1) GVHD symptoms are dependent on SIY antigen expression in the tumor/tissue; 2) GVHD symptoms are affected by the affinity of the TCR transduced into $CD4^+$ cells; 3) Regulatory T cells (Tregs) have a protective role in GVHD symptom manifestation.

To test these hypotheses in a mouse model we used a TCR called 2C, which is derived from a well-characterized CD8+ 2C T cell clone that was originally isolated over 30 years ago by Dr. David Kranz [14]. The 2C TCR recognizes and binds several defined peptide antigens bound to either allogeneic $H\text{-}2L^d$ or syngeneic $H\text{-}2K^b$ class I MHC molecules [15]. In the context of syngeneic class I MHC K^b, 2C cells recognize foreign peptide SIY (SIYRYYGL) with an affinity of 30 μM. In 2002, a high affinity TCR variant of 2C called m33 was isolated in the Kranz lab using the yeast display technique [16]. m33 recognizes SIY-K^b with an affinity of 30 nM, and it is CD8 co-receptor independent [17]. Through TCR gene transfer, it is possible to redirect $CD4^+$ cells to recognize peptides presented by MHC class I molecules.

There are many useful reagents related to this well-defined system. 1b2, a monoclonal antibody against the 2C TCR, can be used to identify 2C T cells by flow cytometry or immunohistochemistry. 2C TCR transgenic mice on the C57BL/6 background express the 2C TCR on approximately 80% or their CD8+ T cells. Tetramer SIY-K^b can be used to detect expression of both 2C and m33 TCR on the cell surface.

4.2 Materials and Methods

Animals

C57BL/6 and C57BL/6 RAG1$^{-/-}$ mice originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were maintained as colonies and housed in the animal facilities at the University of Illinois. Mice were used in experiments when they were 2-5 months old. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois.

Activation and retroviral transduction of primary T cells

T cells were obtained from spleens of C57BL/6 mice and prepared into a single-cell suspension. Erythrocytes were lysed with ammonium chloride (ACK) buffer and CD4$^+$ T cells isolated by magnetic sorting using the Mouse CD4$^+$ T Cell Isolation Kit II (MACS Miltenyi Biotec, Germany). T cell transduction was performed as described in Soto et al. [18]. Plat-E retroviral packaging cells [19] were plated at 4 x 10^6 cells/dish on a poly-L-Lysine (Sigma) coated 10 cm petri dish and cultured for 24 h. Plat-E cells were transfected with 2C or m33 TCR gene, cloned into the pMP71 vector (from myeloproliferative sarcoma virus, MPSV) as 2C beta chain-P2A-2C alpha chain using NotI at the 5'-end and EcoRI at the 3'-end [20]. The 2C TCR gene was codon optimized for expression in murine cells. Plat-E cells were transfected with 40 μg vector DNA, Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen) for 4 h, after which the DNA-transfection mixture was aspirated and 6 ml of cell culture media added to each plate. Viral supernatant was harvested 48 h after transfection, passed through a 0.45 μm syringe filter and 50 μl of Lipofectamine 2000 added per 6 ml of viral supernatant.

1 x 10^6 T cells per well were activated with anti-CD3 and anti-CD28 coated beads (Dynabeads, Invitrogen) and 30 U of recombinant mouse IL-2 (Roche) in a 24 well plate for 24 h. Following activation, Dynabeads were magnetically removed from T cells and cells were transferred into a 24-well plate coated with Retronectin at 15 μg/mL (Takara, Japan). In each well, 1 x 10^6 T cells in 1mL of T cell media were mixed with 60U of recombinant murine IL-2 and 1mL sterile 2C or m33 TCR viral supernatant from transfected Plat-E cells. The plate was centrifuged at 2,000 rpm at 30°C for 1 h. Following centrifugation, cells were incubated at 37°C in 5% CO_2 in a humidified incubator and a second transduction was repeated 24 h later.

Flow cytometry analysis

Lymphocytes from spleens and lymph nodes of mice were prepared by mechanical tissue dissociation through nylon mesh followed by ACK buffer lysis of erythrocytes. Single-cell suspensions were stained for flow cytometry with following reagents: CD4-AlexaFluor647 (BD Pharmingen), Vβ8.1/8.2-PE (BD Pharmigen) and 1b2-biotin (Kranz Lab) antibodies; SIY-K^b-PE tetramer (Kranz Lab) and Strepavidin-APC

(Invitrogen). All reagents were used at final concentration of 10 μg/ml for 1 hr at 4°C, and washed with with 0.5% PBS/BSA in between staining steps. Cells were analysed on the Accuri C6 instrument and FCS Express software.

Tissue sections and immunostaining

After the mice were euthanized, their ears, parts of intestines and lymph nodes were isolated and snap-frozen in OCT medium for cryosectioning and immunostaining. Eight μm cryosections were taken. Primary antibodies used for staining were: rabbit anti-CD3 (Abcam, Cambridge, MA) and rat anti-FoxP3 (eBioscience, San Diego, CA). Secondary antibodies used: donkey anti-rabbit-DyLight 488 and donkey anti-rat-DyLight 594 (Jackson ImmunoResearch, West Grove, PA). For immunostaining, slides were fixed in cold 95% ethanol and blocked with Superblock (Thermo Scientific, Rockford IL). Sections were then incubated with a primary antibody in PBS + 20% glycerol (PBSG) overnight, washed with PBS + 0.1% Tween-20 (PBST), and incubated with secondary antibody in PBST for 4 h. Slides were washed and briefly incubated with DAPI (Invitrogen, Carlsbad, CA). Control slides omitting the primary antibody were negative for DyLight 594 or DyLight 488. Images were obtained with an Olympus BX-51 microscope at 20x magnification.

Data Analysis

GraphPad Prism software (La Jolla, CA) was used for all statistical analyses and graph presentation. Symptom onset data were recorded from the time of the adoptive transfer until symptoms were first observed and were plotted using a Kaplan-Meier curve. Significance was considered $P<0.05$.

4.3 Results

Onset of GVHD-like symptoms is independent of the SIY antigen

Previous occurrences of GVHD-like symptoms observed in our laboratory were a side effect of therapy with T cells genetically modified to recognize the model antigen SIY present on tumors in mice. Treatment with T cells transduced to express TCRs that are specific for SIY peptide resulted in prolonged survival in melanoma and glioma models [18] [Soto CM, unpublished data]. However, it was unclear whether the presence of the foreign SIY antigen was necessary to drive the GVHD-like side-effects caused by the T cells.

In order to test this requirement, tumor-free $RAG1^{-/-}$ mice were injected with $CD4^+$ T cells transduced either with 2C TCR, m33 TCR or mock transduced (n=3 per group). $CD4^+$ cells were enriched from primary lymphocytes isolated from C57BL/6 mice. Cells were then activated and retrovirally transduced with the SIY-specific 2C TCR, its high-affinity version m33 TCR or mock transduced (Figure 4.1). Approximately 6.5×10^6 T cells were administered through tail vein injection per mouse.

Mice were monitored for 70 days for development of GVHD-like symptoms (skin lesions associated with ears and face, tails and limbs, ulceration, weight loss). After 70 days, mice were injected with 10 µg of SIY peptide i.p., which is a treatment that activates 2C T cells in vivo [21]. One mouse from each group was left as an untreated control, and the rest were further observed for signs of GVHD-like symptom progression.

By day 70, when challenge with SIY peptide i.p. was planned and executed, GVHD symptoms were observed in some of the treated mice (Figure 4.2). Figure 4.2A shows a timeline of the symptom onset per group in the Kaplan–Meier curve format. There was a significant effect of TCR transfection on symptom onset time ($p < 0.01$). Images of experimental animals are shown in Figure 4.2B, and GVHD lesions are evident in affected mice. Table 4.1 summarizes the details of symptom onset, symptom description and experimental manipulation. Development of GVHD symptoms in experimental mice was independent of the presence of the SIY antigen in the system: symptoms developed without SIY involvement and did not worsen after exposure to SIY.

Affinity of the transduced TCR as a factor in manifestation of GVHD-like symptoms

From previous experiments, it was clear that onset of GVHD symptoms was correlated with the transfer of genetically modified $CD4^+$ T cells. However, the contribution of the affinity of the transferred TCR for its target was inconclusive. In our experiment, in the 2C $CD4^+$ group, all three animals developed symptoms, and one animal had to be euthanized due to 25% weight loss at day 40 post ACT. In the m33 $CD4^+$ group,

two out of three animals developed lesions on the ears. In the mock transduced group, no GVHD symptoms were observed (Figure 4.2). Treatment with $CD4^+$ T cells transduced with 2C TCR showed a trend towards quicker development and worse manifestation of GVHD symptoms as compared to m33 TCR-transduced cells. This is consistent with earlier observations in tumor-bearing mice. Our results suggest that GVHD symptoms observed in our model are TCR and self-antigen dependent, but the influence of affinity of the transferred TCR requires further investigation.

TCR transduced $CD4^+$ cells persist in mice with GVHD-like symptoms

After the mice were euthanized, we examined the persistence of transferred $CD4^+$ cells in $RAG1^{-/-}$ mice. One mouse had to be euthanized early due to the severity of GVHD symptoms (day 40 post ACT), while the other were kept for 4-5 months after the cell transfer.

In order to test initial transduction efficiency of murine $CD4^+$ T cells with 2C and m33 TCRs, we stained the cells with SIY-K^b tetramer that binds to both 2C and m33 TCRs (Figure 4.1A), as well as with the antibody for Vβ8.1,8.2 TCR chains present in both transduced TCRs (Figure 4.1B). According to the SIY-K^b tetramer binding, transduction efficiency was 36% and 32% for 2C and m33, respectively in $CD4^+$ cells (background staining of mock transduced cells shows 1%). Similar values are obtained from staining for presence of Vβ8.1,8.2 TCR chain. 41% of primary murine lymphocytes express the same TCR Vβ, as evident in the histogram of mock transduced cells.

Figure 4.3 shows splenocytes from the mouse 1682 that received 2C $CD4^+$ T cells and had to be euthanized due to the severity of symptoms 40 days after cell transfer. Cells are stained with 1b2, a clonotypic antibody for 2C TCR and show persistence of substantial amount of 2C+ cells (approximately 60% of all $CD4^+$ splenocytes) at this early time-point (Figure 4.3A). There were 75% Vβ8.1,8.2 TCR positive splenocytes in mouse 1682 (Figure 4.3B), which correlates well with 72% $V\beta^+$ transferred 2C TCR transduced T cells (Figure 4.1B).

When the experiment was terminated after 5 months, splenocytes isolated from remaining mice were also evaluated for persistence of transferred TCRs, by staining with SIY-K^b tetramer, 1b2 clonotypic antibody for 2C TCR and the more general Vβ8.1,8.2 TCR stain (Figure 4.4). Tetramer stain of the $CD4^+$ splenocyte subset was positive for 24% and 16% cells in the 2C group mice and for 26%, 31% and 17% cells in the m33 group mice (background staining of mock transduced cells was ~2%) (Figure 4.4A). Stain with 1b2, expected to stain only 2C TCR, stained 43% and 36% $CD4^+$ cells in two mice from the 2C group, with background staining of about 6% (Figure 4.4B). $V\beta^+$ fraction of all splenocytes was 8% and 10% for mice that received mock transduced cells, 19% and 25% for 2C TCR recipients and 14%, 27% and 21% for m33

TCR recipients (Figure 4.4C). These results show sustained persistence of transferred genetically modified T cells, as far as 5 months after adoptive transfer.

Presence of regulatory T cells in organs of treated mice shows no strong correlation with symptom presence

We hypothesized that regulatory T cell phenotype (Treg) may have a protective role in the context of GVHD symptom development in our model. We evaluated the presence of Tregs in the organs affected by GVHD symptoms by immunohistochemistry. Frozen sections of lymph nodes, ears and intestines were sectioned and stained for the universal T cell marker, CD3, as well as for the Treg marker, transcription factor FoxP3. Representative sections of analyzed organs are presented in Figure 4.5; the top panels show examples of sections from mice affected by GVHD symptoms, and the bottom panels are from mice that remained symptom-free. In our sample, there was no strong correlation of Treg presence and protection from GVHD symptom onset. Lymph node sections of all analyzed mice were abundant with FoxP3+ cells (Figure 4.5A, red). Intestine from 1683, the protected mouse from the m33 TCR group, suggested an increased Treg infiltration compared to other samples (Figure 4.5B). However, most of the Treg infiltration in the ears was observed in 1692, a 2C recipient mouse with noticeable, albeit controlled ear damage.

4.4 Discussion

Our results suggest that GVHD-like symptoms observed in our model are TCR and self-antigen dependent. Symptoms did not manifest in mice treated with mock transduced $CD4^+$ cells, and were independent of TCR-specific antigen (SIY) expression in the host. There was a trend towards more severe and earlier onset symptom manifestation associated with treatment with cells transduced with the wild type TCR (2C) compared to the high affinity TCR (m33). This is consistent with prior observations in our laboratory, in the context of tumor-bearing mice [Soto CM, unpublished data]. In these prior studies, effects appeared to be more predominant with 2C TCR in $CD4^+$ T cells than m33 in $CD4^+$ T cells, and the most severe GVHD clinical signs were exhibited in mice which received a combination treatment of CD8+ and $CD4^+$ cells, suggesting that CD8+ T cells might exacerbate symptoms induced by $CD4^+$ cells [Soto CM, unpublished data].

Experimental evidence in mice has shown that new self-reactive T-cell specificities created upon the formation of mixed TCR dimers following transfection with TCR genes can result in autoimmune destruction [22] [23]. This pathology, termed "TCR gene therapy-induced graft-versus-host disease," only becomes apparent under conditions in which the TCR-modified T-cell response is vigorous [22]. Since similar findings have not been found (or reported) in ACT clinical trials done in humans, speculations were present that it is a problem unique to mice [24]. It is possible however that the problem will occur with increasing the *in vivo* function of TCR-modified T cells in humans, and it has been demonstrated with human cells *in vitro* [23]. Two TCR engineering strategies can be used to combat the observed mixed TCR dimer-dependent GVHD symptoms in mice. First one is the use of TCR engineered with an additional inter-chain disulphide bond [25], and the other one is linking TCR-α and TCR-β genes with a virus-derived P2A element [26]. These strategies can limit or prevent GVHD symptoms in mice after TCR gene transfer [22] and might also enhance the antitumor efficacy of TCR gene therapy in mice [4]. GVHD-like symptoms observed in our lab are unlikely due to TCR α and β chain mis-pairing (formation of "mixed TCT dimers") as these TCRs have been codon optimized, contain the P2A cleavage site, and the extra C region cysteines that have been shown to mitigate the mis-pairing problems [4].

Given that many studies have shown that transfer of $CD4^+$ T cells from C57BL/6 mice into $RAG1^{-/-}$ mice induces GVHD-like symptoms [9] [10], the surprising result in our studies was the lack of GVHD-like symptoms in mice treated with mock transfected T cells. $CD4^+$ T cells are able to induce GVHD symptoms even in mice that do not express MHC II [9]. The cellular interactions between $CD4^+$ T cells and macrophages may be critical for the induction of GVHD; for example, manipulation of TLR-4 agonists in the gut (by altering the intestinal flora) affects whether $CD4^+$ T cells induce GVHD-like symptoms or have no effect [27]. Other studies have shown that polymorphisms in macrophage pattern recognition receptors

affect whether $CD4^+$ T cells induce GVHD-like symptoms. Interestingly, the subtype of $CD4^+$ cells that most effectively induces intestinal damage is the $CD45RB^{high}$ subset, which is characteristic of naïve $CD4^+$ T cells [10]. Co-injection of $CD45RB^{low}$ $CD4^+$ T cells prevents the induction of symptoms [28]. Perhaps there is some low affinity interaction of the transfected TCR with self-antigens that allows maintenance of a more naïve phenotype, which does not occur with the mock transfection.

We were unfortunately unable to shed much light on the cause of the GVHD-like symptoms in mice treated with $CD4^+$ transfected T cells. Injection of $CD4^+$ T cells has been used to create models of colitis and GVHD-like symptoms for more than 20 years, and the roles of multiple cell types in the response are still being investigated.

4.5 References

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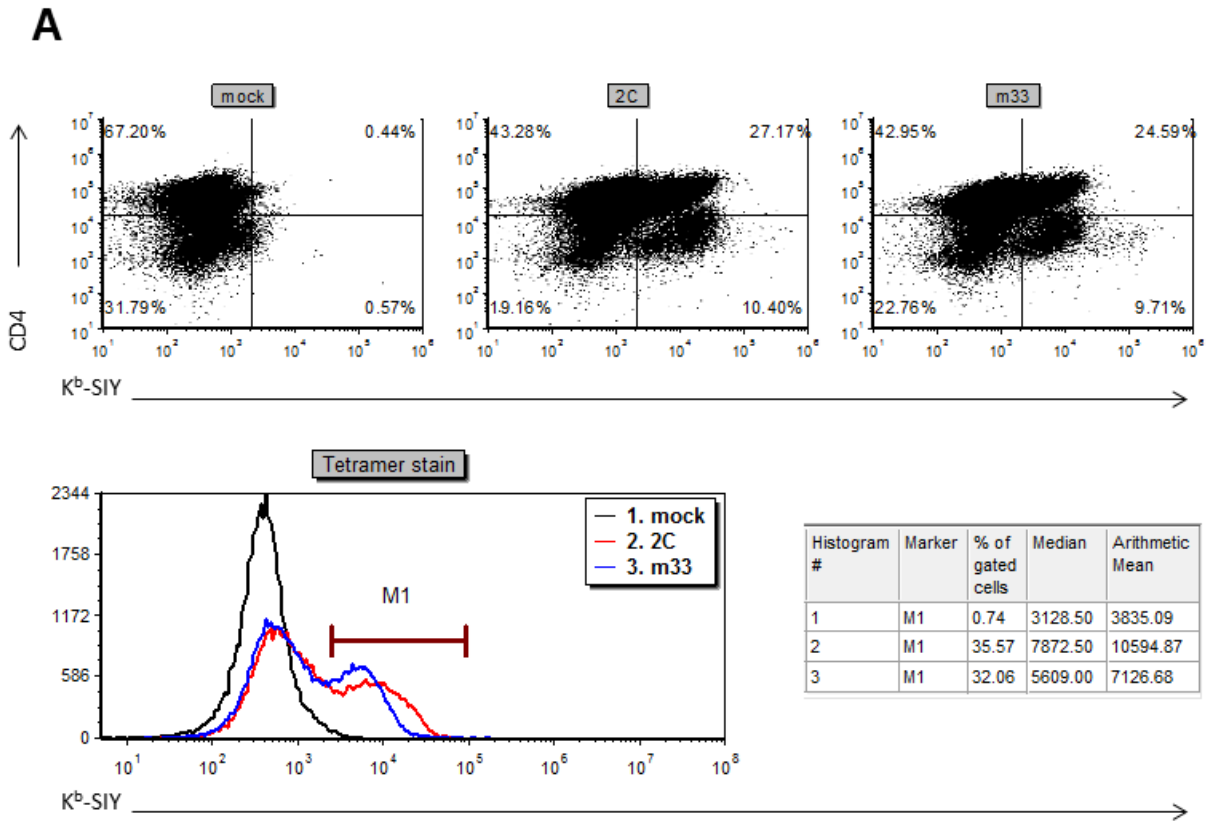
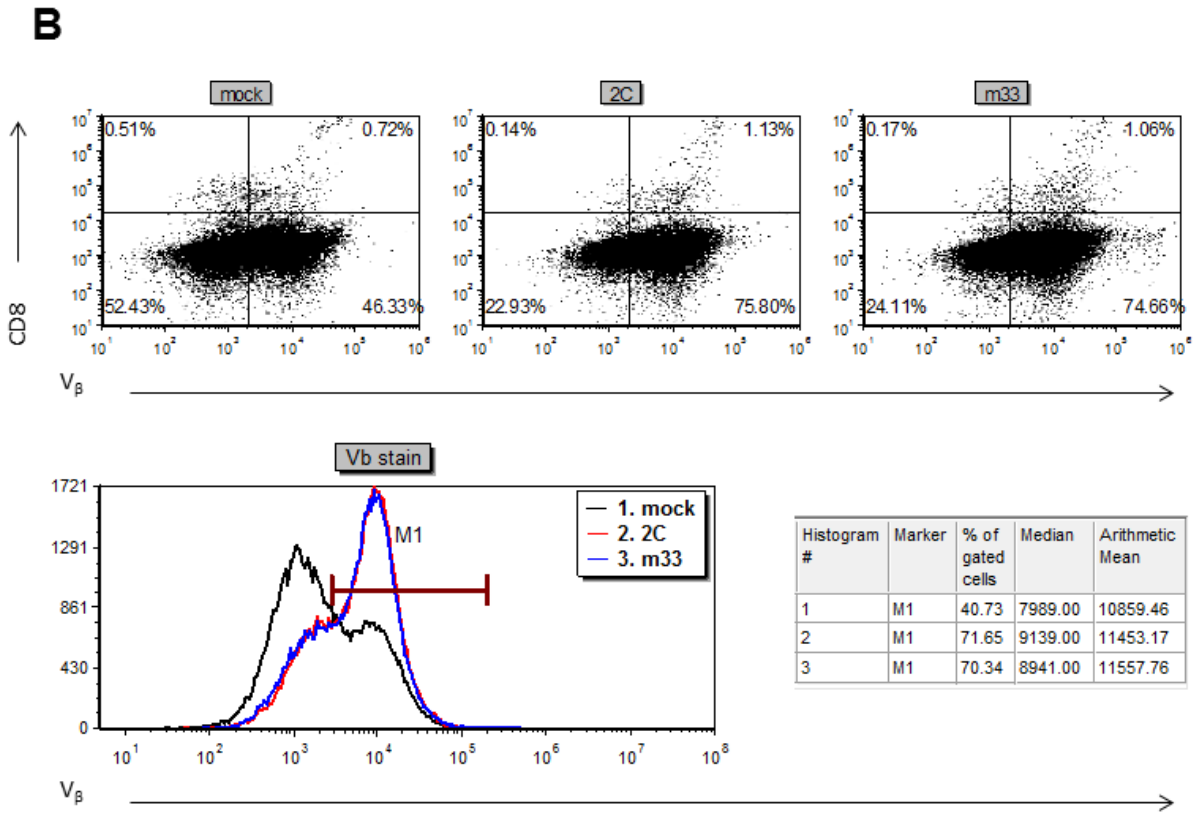
4.6 Figures



Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	0.74	3128.50	3835.09
2	M1	35.57	7872.50	10594.87
3	M1	32.06	5609.00	7126.68

Figure 4.1 – Phenotype of transduced CD4^{+} splenocytes prior to adoptive transfer to RAG1$^{-/-}$ mice. CD4+ T cells were isolated by magnetic sorting from C57BL/6 mice splenocytes and retrovirally transduced with 2C TCR, m33 TCR or mock transduced. (A) Dot plots showing TCR-transduced cells positive for CD4 and K^{b}-SIY tetramer, which stains both 2C and m33 TCR; Histograms showing the K^{b}-SIY tetramer stain for all conditions. (B) Dot plots showing TCR-transduced cells positive for CD8 and TCR β chain Vβ8.1,8.2, which is present in both 2C and m33 TCR, and some endogenous TCRs; Histograms showing the Vβ stain for all conditions. Percentages in tables indicate fraction of gated cells in the designated M1 marker (cells considered positive).



Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	40.73	7989.00	10859.46
2	M1	71.65	9139.00	11453.17
3	M1	70.34	8941.00	11557.76

Figure 4.1 (cont.) – Phenotype of transduced CD4^{+} splenocytes prior to adoptive transfer to RAG1$^{-/-}$ mice.

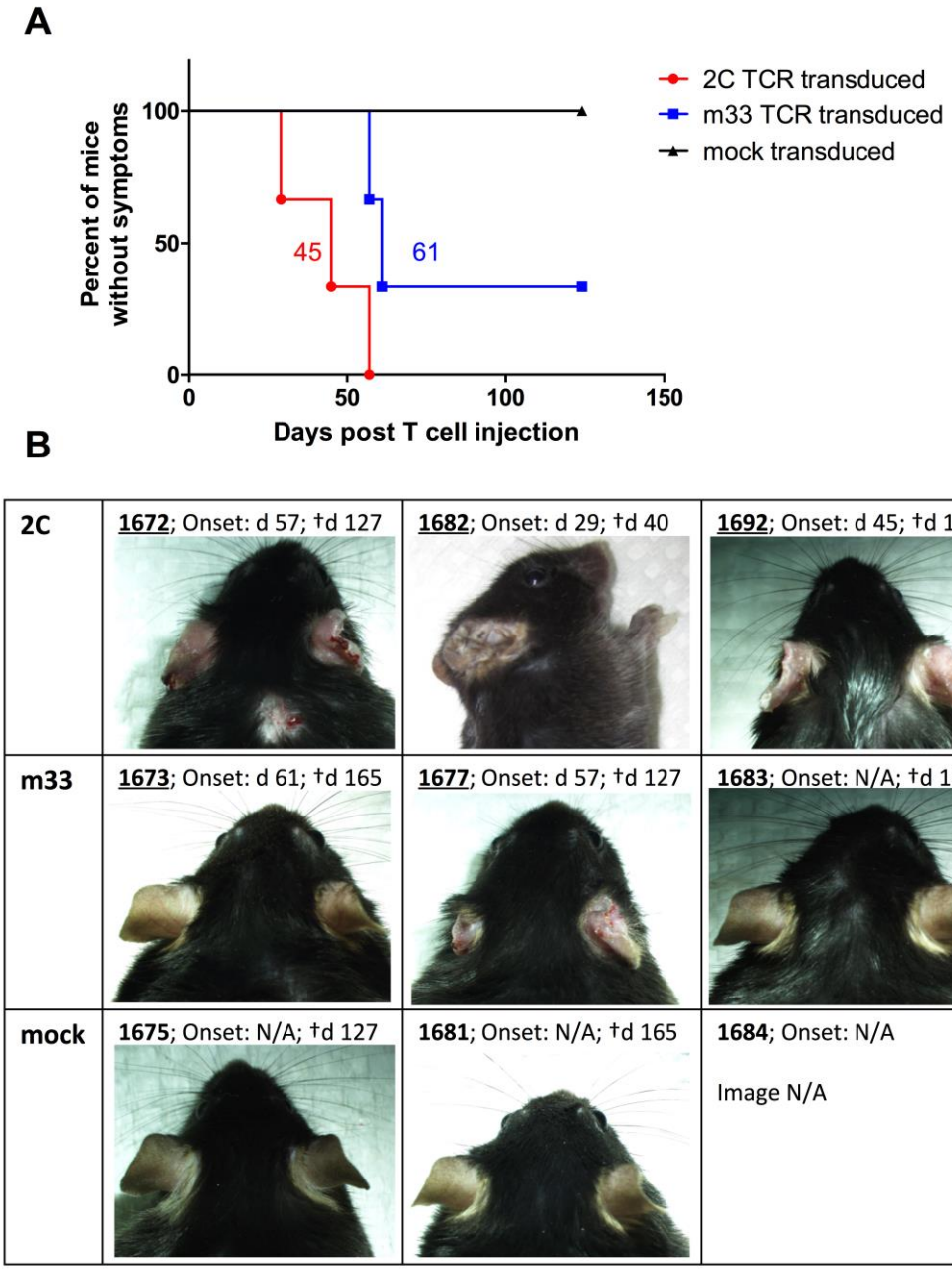


Figure 4.2 – GVHD symptoms as a result of auto-reactive CD4$^+$ T cells transduced with different TCRs in RAG1$^{-/-}$ mice without tumors. RAG1$^{-/-}$ mice were injected with CD4$^+$ cells transduced with 2C TCR, m33 TCR or mock transduced. Mice were monitored for onset of GVHD symptoms (skin lesions on ears, face, tails and limbs, ulceration, weight loss). (A) Kaplan–Meier curve presenting percent of mice that have developed GVHD symptoms. Numbers on graph indicate median day of symptom onset post-adoptive cell transfer. Number of mice was 3 per group. (B) Images of physical GVHD presentation on mice following transduced CD4$^+$ T cell injection. All animals were euthanized on day 127 or day 165 post-cell transfer, apart from one animal that had to be sacrificed at day 40 because of weight loss. GVHD signs include skin lesions on ears and head (shown), tails and limbs of mice (not shown). Underlined mouse numbers indicate animals that developed GVHD symptoms.

Mouse	Group	GVHD symptoms	Day of onset	Day of medical case report	Day euthanized	Details	SIY i.p. day 70:
1672	2C	Yes	57	82	127	Crusty ears.	SIY
1682	2C	Yes	29	40	40	Crusty ears and tail.	n/a
1692	2C	Yes	45	n/a	165	Crusty ears. Jittery.	PBS
1673	m33	Yes	61	n/a	165	Red rim on ears. Symptoms resolved later.	SIY
1677	m33	Yes	57	100	127	Red rim on ears.	PBS
1683	m33	No	n/a	n/a	165		SIY
1675	mock	No	n/a	n/a	127		SIY
1681	mock	No	n/a	n/a	165		SIY
1684	mock	No	n/a	n/a	165		PBS

Table 4.1 – Summary of GVHD symptoms in RAG1$^{-/-}$ mice following transfer of transduced CD4^{+} T cells

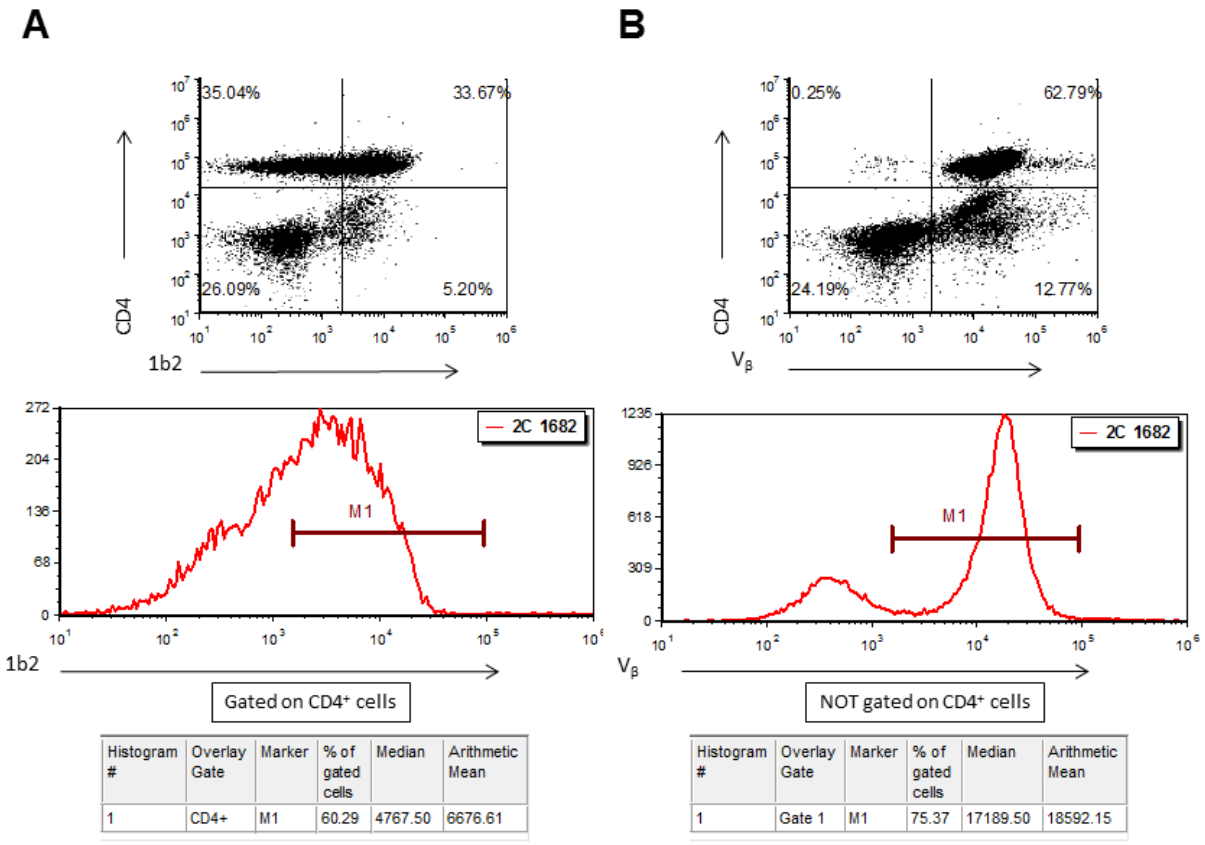
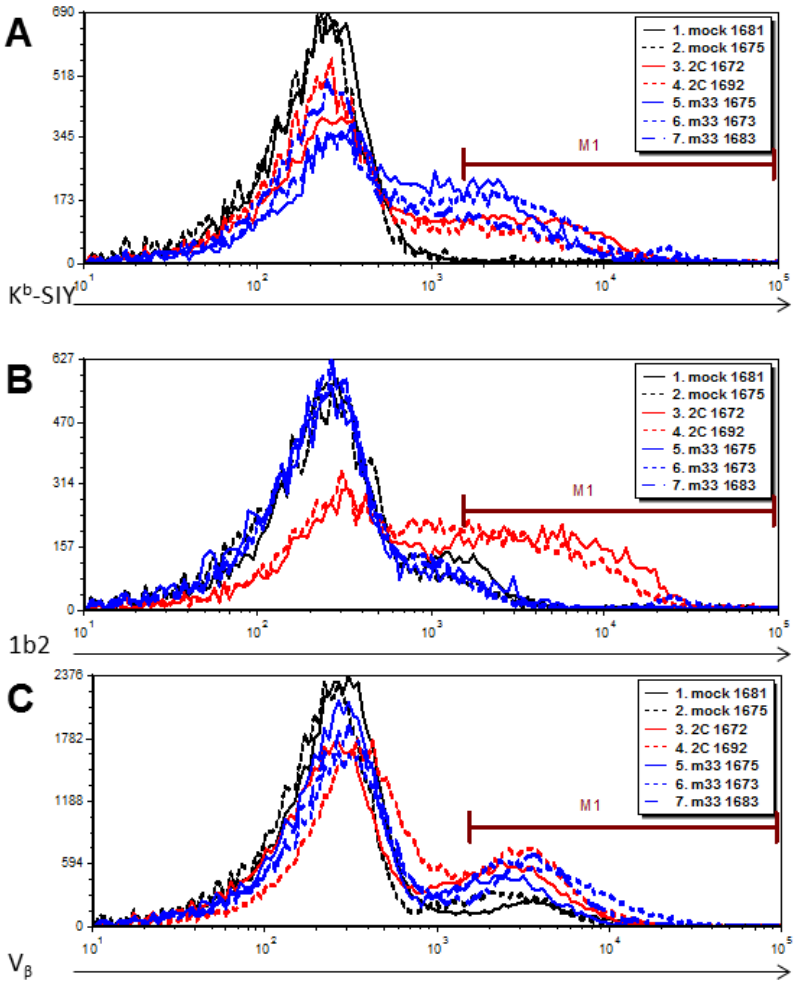


Figure 4.3 – Phenotype and persistence of T cells transduced with 2C TCR in the mouse with most acute GVHD symptoms at day 40 post adoptive transfer. On day 40 post adoptive transfer, mouse 1682 that got $CD4^+$ T cells transduced with 2C TCR needed to be euthanized because of weight loss. The spleen was isolated and persistence of transferred cells was analyzed. (A) Dot plot showing CD4 and 2C TCR positive cells (stained by clonotypic 1b2 antibody); histogram is gated on $CD4^+$ cells. (B) Dot plot showing CD4 and TCR Vβ8.1,8.2 levels; histogram is gated the same way as the dot plot. Percentages in tables indicate fraction of gated cells in the designated M1 marker (cells considered positive).



Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	1.51	7615.00	14994.30
2	M1	2.15	5343.00	11229.14
3	M1	24.44	4200.00	5949.43
4	M1	15.67	3675.00	6477.78
5	M1	25.62	2843.50	4930.28
6	M1	31.09	3671.00	6182.11
7	M1	16.71	3240.50	5342.77

Gated on CD4+ cells

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	7.75	2189.00	6597.31
2	M1	5.95	2620.00	9902.83
3	M1	43.08	5172.50	7346.93
4	M1	35.93	4152.00	6584.10
5	M1	7.30	2735.50	8121.83
6	M1	5.94	3684.00	13148.46
7	M1	4.64	2216.00	6638.20

Gated on CD4+ cells

Histogram #	Marker	% of gated cells	Median	Arithmetic Mean
1	M1	8.11	3755.00	4794.92
2	M1	9.57	2963.00	3588.33
3	M1	18.84	3126.50	3785.35
4	M1	24.94	3411.00	4221.63
5	M1	14.17	2925.00	3522.24
6	M1	27.06	4062.50	5820.42
7	M1	21.26	3770.00	4656.00

NOT gated on CD4+ cells

Figure 4.4 – Phenotype and persistence of transduced T cells in mice 4 and 5 months post ACT. On days 127 or 165 post adoptive transfer, mice that got CD4$^+$ T cells transduced with 2C, m33 or mock TCR were euthanized, their splenocytes were isolated and tested for persistence of transferred cells. Histograms from all remaining mice are overlaid for each stain. Black histograms – mock, red histograms – 2C, blue histograms – m33 (A) K^b-SIY tetramer stain of cells from all remaining mice. Histograms are gated on CD4$^+$ cells. (B) 1b2 (clonotypic antibody for 2C TCR) stain of cells from all remaining mice. Histograms are gated on CD4$^+$ cells. (C) TCR Vβ8.1,8.2 levels of cells from all remaining mice. Histograms are gated on all lymphocytes. Percentages in tables indicate fraction of gated cells in the designated M1 marker (cells considered positive).

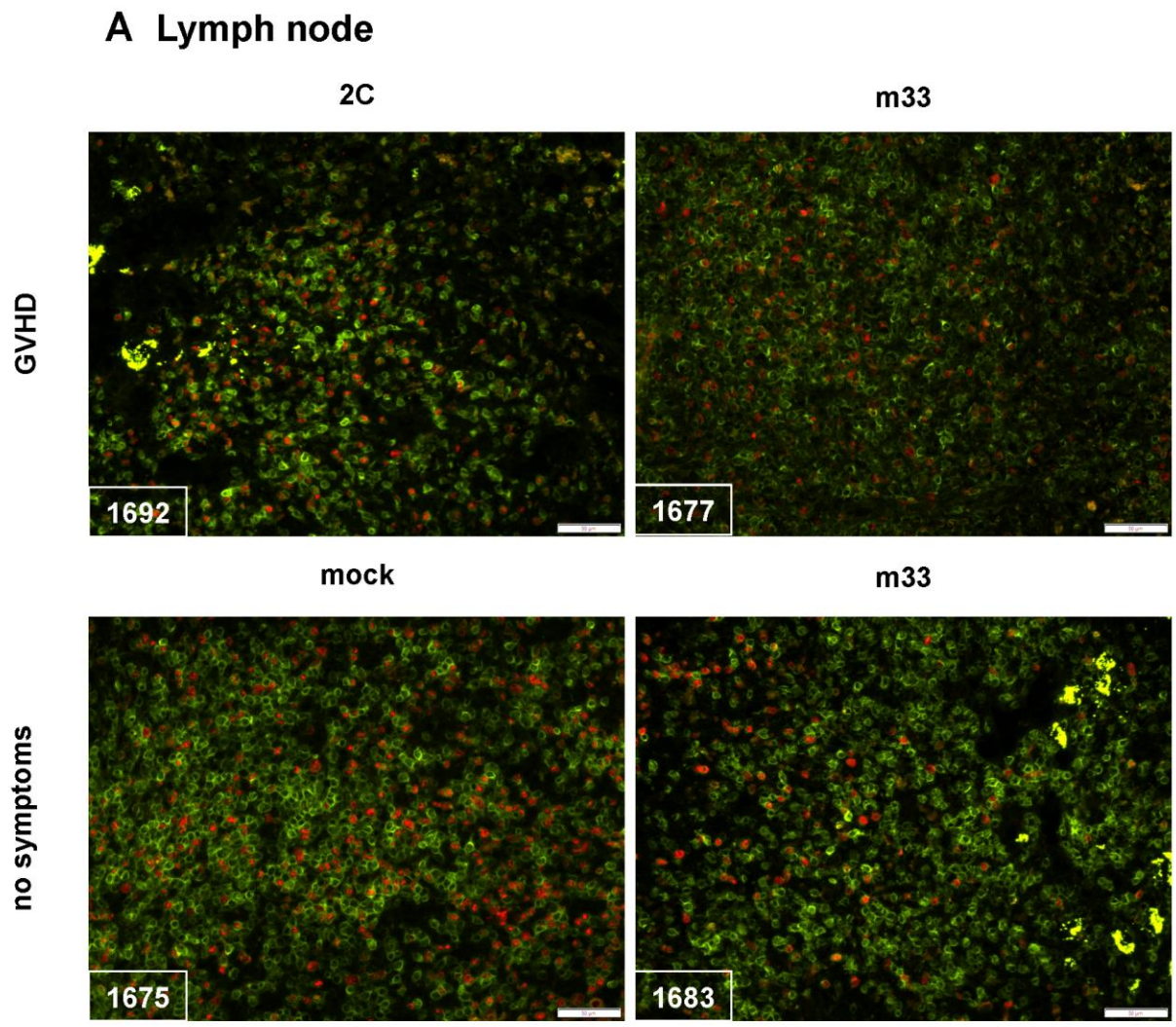


Figure 4.5 – Presence of regulatory T cells in organs of mice that received transduced T cells. RAG1$^{-/-}$ mice were injected with CD4^{+} cells transduced with 2C TCR, m33 TCR or mock transduced. Mice were monitored for onset of GVHD symptoms (skin lesions on ears, face, tails and limbs, ulceration, weight loss). On days 127 or 165 post adoptive transfer, mice were euthanized, their lymph nodes, parts of intestine and ears were isolated, frozen in embedding media, and cryosectioned. Organ sections were immunostained for CD3 surface marker (T cells) and FoxP3 transcription factor (Tregs). Representative organ sections are shown; top panels show examples of sections from mice affected by GVHD symptoms, and bottom panels are from mice that remained symptom-free. Treatment group is indicated above the image, and mouse ID is embedded in the image. (A) Lymph node. (B) Intestine. (C) Ear. Green – CD3-positive, red – FoxP3-positive. Scale bar = 50 micrometers.

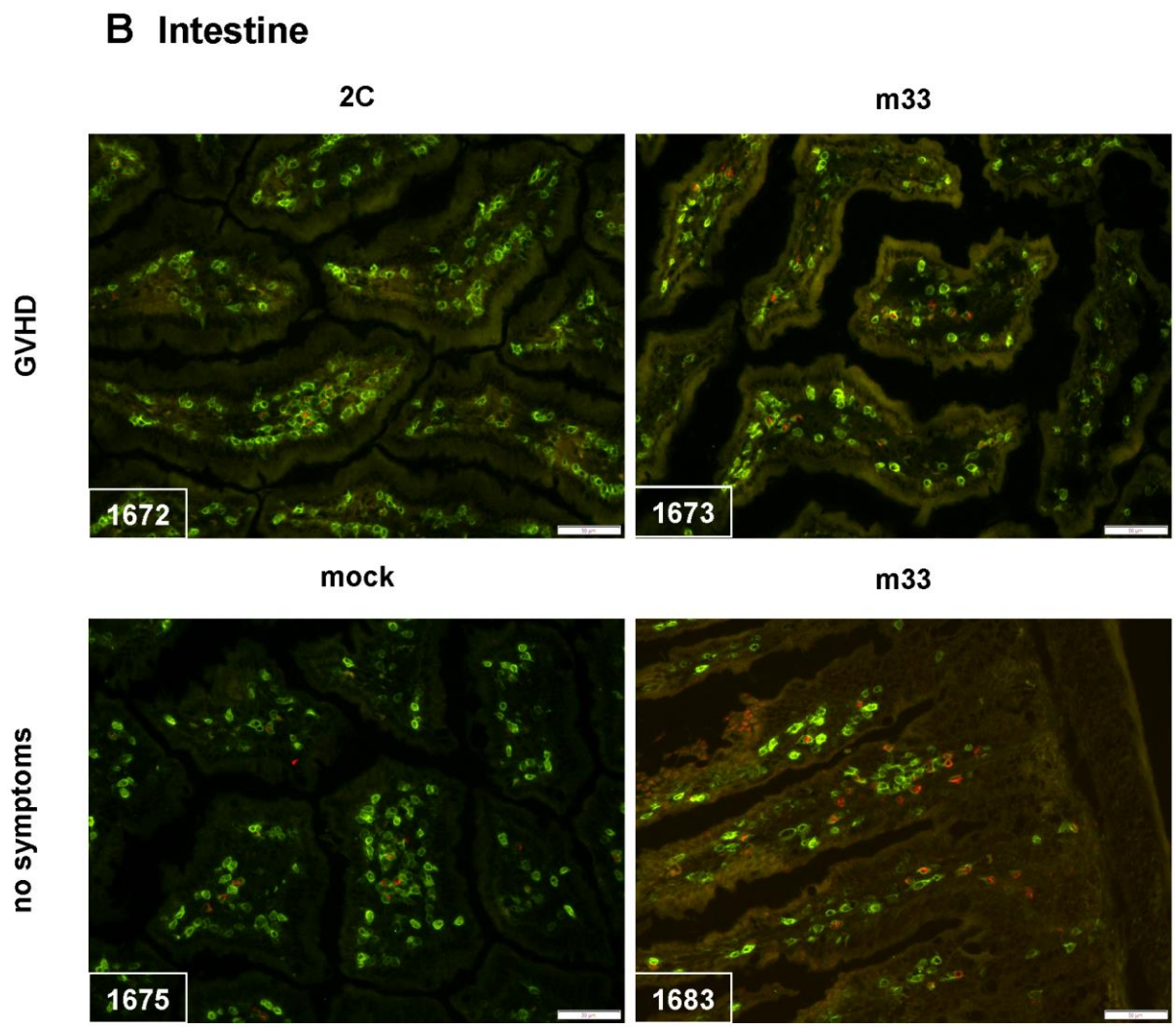


Figure 4.5 (cont.) – Presence of regulatory T cells in organs of mice that received transduced T cells.

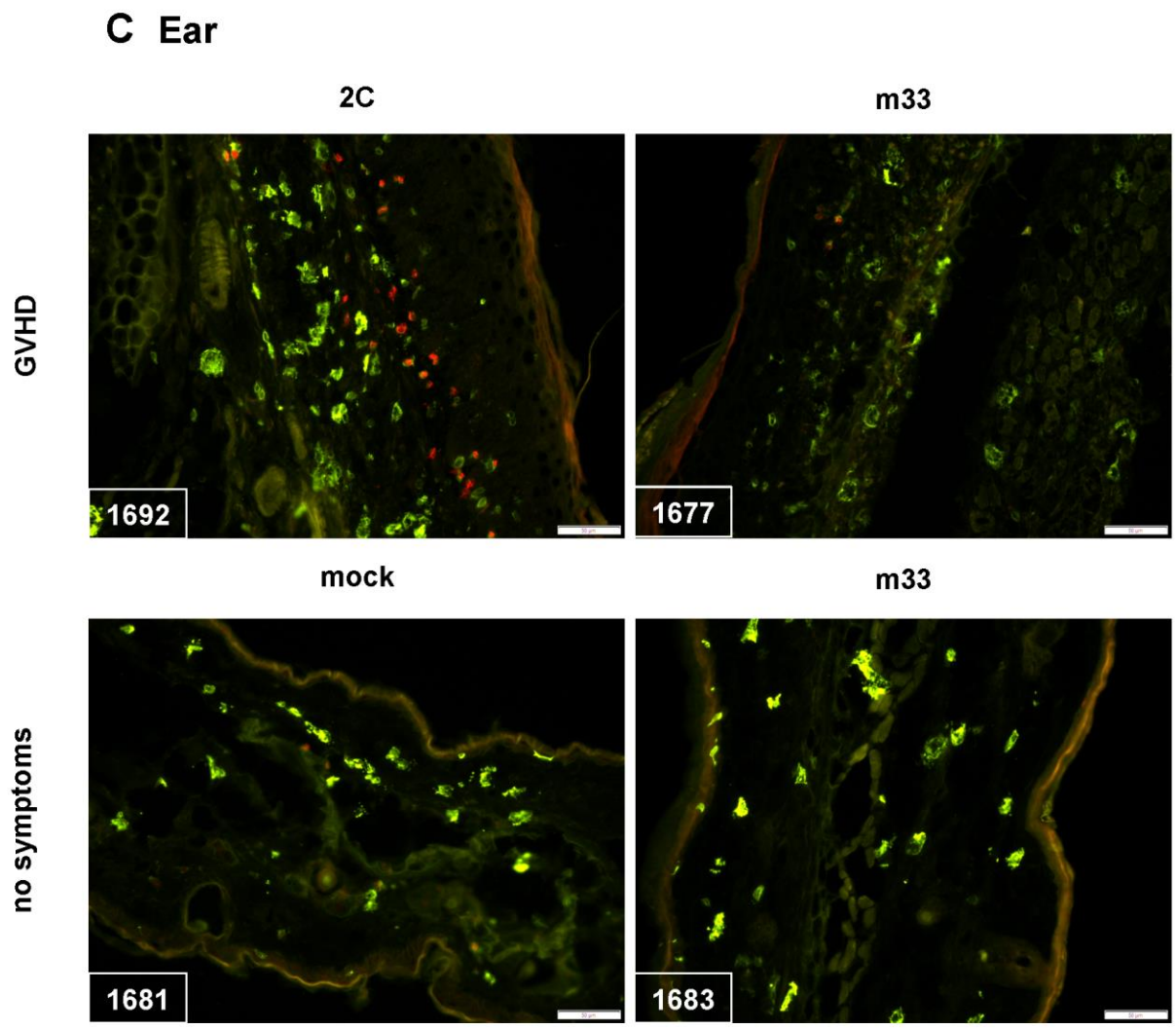


Figure 4.5 (cont.) – Presence of regulatory T cells in organs of mice that received transduced T cells.

CHAPTER FIVE
CONCLUSION

Immunotherapies and especially adoptive cell transfer are emerging as therapeutic modalities with potential for high impact in treatment for cancers and other chronic diseases. It is likely that immunotherapy, like other anti-cancer strategies before it, will benefit from some form of combination therapy in order to achieve the greatest clinical outcome. Oncolytic viral therapy, the use of cancer-specific viruses to target and kill cancer cells, is a therapeutic modality that is well suited to deliver and augment immunotherapeutic strategies.

The aim of my thesis was to improve efficacy and safety of antitumor immunotherapy. We explored several approaches to achieve this goal. Firstly, we have analyzed antitumor effects of an oncolytic myxoma virus armed to secrete an immunostimulatory protein at the tumor site. In order to do that, we engineered a new recombinant myxoma virus (vMyx-IL15Rα-tdTr), which expresses IL15Rα-IL15 fusion protein. We described the therapeutic effects observed with the new virus in a mouse model of aggressive melanoma, B16-F10, in immune deficient as well as immune competent mice. Treatment with the novel virus resulted in a significant survival benefit for the treated group compared to the controls, and immunohistological analysis suggested that both the innate and adaptive components of the host immune system play a role in the antitumor effect. Our work contributes to the growing body of evidence that using armed viruses to boost antitumor immune response is a feasible strategy for complementing immunotherapies.

In order to further expand this approach, we explored the use of myxoma virus for increasing tumor antigen presentation at the tumor site. Tumor-antigen specific therapies face the challenge of selecting for subpopulations of cancer cells that no longer express the target and cause reemergence of the tumor. We designed a model-tumor antigen expressing myxoma virus, for the purpose of enhancing combination therapy with antigen-specific T lymphocytes. We hypothesize that the combination of the viral effect of enhancing the attenuated immune response and increasing the presence of T cell specific antigen in the tumor would lead to tumor elimination.

With the emergence of adoptive T cell therapy as a more widely used treatment modality, the safety of this groundbreaking approach will be a major concern. Severe side effects of target-specific T cell therapy that are emerging in clinic due to cross-reactivity of transferred cells with normal host tissues were foreshadowed by preclinical studies. We have observed unexplained graft-versus-host like symptoms as a side effect of adoptive T cell cancer therapy in mice in our laboratory when CD4+ T cells are used. In order to gain insight into the cause of the symptoms, we examined the outcome of transfer of genetically modified T cells to tumor-free recipients and analyzed the underlying pathology. There is still a lot left to investigate

in this phenomenon: the role of multiple cell types in the response, as well as the contribution of the genetic modification and of cell receptors engineered for higher affinity.

Together, these studies have made a significant contribution to the general field of combination immunotherapy. The most notable result of my work is proof of principle that viral delivery of IL15Rα-IL15 can improve the antitumor effect, and cause dramatic recruitment of immune cells to the tumor site. A combination of different delivery vehicles for the IL15Rα-IL15 fusion protein should be explored in the future. Also, viral delivery could be improved by combination treatment with drugs such as rapamycin. In order to make the most out of the viral specificity for the cancer cells, improving systemic delivery of the virus for treatment of metastatic disease would be a big improvement in this proposed strategy.